MICRORNA SIGNATURES IN PLANT GENOME STABILITY AND GENOTOXIC STRESS

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MICRORNA SIGNATURES IN PLANT GENOME STABILITY AND GENOTOXIC STRESS

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Editorial: MicroRNA Signatures in Plant Genome Stability and Genotoxic Stress

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Editorial on the Research Topic

MicroRNA Signatures in Plant Genome Stability and Genotoxic Stress

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Plants are subjected to high levels of DNA damage resulting from their essential dependence on sunlight and exposure to environmental stresses. Accumulation of DNA lesions generated by genotoxic stress disturb genome stability, which hinders plant development and crop productivity. To ensure the maintenance of genome stability, plants developed a range of mechanisms aiming at detecting and repairing DNA damage. The intricated DNA damage response (DDR) network consists of an impressive array of DNA damage sensing and signal transduction pathways leading to DNA repair and cell survival or, alternatively, triggering cell death. Although DDR is highly conserved in eukaryotes, peculiar plant-specific features are described (Yoshiyama et al., 2013; Nikitaki et al., 2018; Nisa et al., 2019). DDR is generally far less studied in plants as compared to mammals (Gimenez and Manzano-Agugliaro, 2017). Due to the essential role of DNA repair in maintaining genomic stability, tightly controlled regulatory mechanism are required, where different players, including phytohormones (Donà et al., 2013) or epigenetic regulators (Kim, 2019), are implicated.

The role played by miRNAs on the post-transcriptional regulation of the DDR has been less considered, particularly in plants when compared to animal systems. Indeed, miRNAs-mediated regulation of DDR gene expression has been already demonstrated in mammalian systems, highly explored in view of therapeutic applications (He et al., 2016; Majidinia and Yousefi, 2016; Rezaeian et al., 2020). In plants, the role of miRNAs in the regulation of DNA damage sensing and repair mechanisms remains to be elucidated.

The present collection of articles gathered within the scope of this Research Topic aims to focus on the implication of miRNAs in genome integrity and response to genotoxic stresses, through direct or indirect interactions with DDR components.

In an extensive review article, Cimini et al. discussed the interconnections between DDR and redox systems, painting a dynamic picture intertwined with regulatory mechanism mediated by miRNAs. Based on their literature analysis, the authors propose a triangular model for redox balance, DDR and miRNAs, where reactive oxygen species (ROS) act as common denominators. At the level of the nucleus, accumulation of ROS results in excessive DNA damage and cell cycle inhibition whereas DDR aid plants to cope with these alterations. Disturbances in the antioxidant and oxidant balance can influence cell cycle progression. Hence, ROS and redox signals are involved

in the regulation of gene expression at transcriptional (redox-sensitive transcription factors) and post-transcriptional (miRNAs) levels. The manuscript enlists a series of miRNAs targeting genes involved in ROS production and scavenging along with putative miRNAs implicated in DDR-associated pathways. While more miRNAs associated with ROS metabolism have been experimentally tested, in the case of DDR most examples arrive from *in silico* studies.

Similarly, the review work by Chowdhury and Basak reinforce the fact that only few plant miRNAs have been identified as active players in combating genotoxic stresses and underlines specific challenges related to miRNA research in this context. As previously mentioned, the lack of in-depth information is a consequence of DDR being significantly less studied in plants compared to animals (Hurdle I) and this combines with the limited information on miRNA targets specifically involved in coping with genotoxic stress (Hurdle II). To address these hurdles, the authors encourage the development of more focused experimental designs combined with the application of omics techniques.

Differently, the review work by Valdés-López et al. provides a different vision on the topic, by looking into the Argonaute (AGO) proteins and their implications in the symbiotic relations between bacteria and legumes, with a stopover on DNA damage/repair-related issues. Being a highly complex process, the regulation of legume-rhizobia symbiosis (LRS) is genetically controlled while some miRNAs are known to play active roles in the post-transcriptional regulation of LRS. AGO proteins (key players in all small-RNA-guided gene-silencing processes) also appear to be involved in LRS, supported by evidence that legumes possess more genes coding for these proteins compared to other non-symbiotic species. In relation to DDR, it is underlined that during rhizobial infection, nodule cells are subjected to endoreduplication, a plant-specific response to DNA damage, cell-cycle arrest, and cell death. Weather AGO proteins with known roles in DNA repair (AGO2, AGO9) are involved in this specific process, still remains to be elucidated.

The "xenomiR" hypothesis proposes that miRNAs can be transferred from one species to another and potentially target genes across distant species. The research work by Bellato et al. combine this cross-kingdom topic with the evolutionary conserved DDR pathway aiming to answer if plant miRNAs could target DDR-related processes in both plant and human cells. The authors have developed a series of bioinformatic approaches to investigate and compare miRNA targets, attesting that these methodologies can be standardized for different species whereas the generated results can serve as starting point for experimental validation of such data. This work succeeded to identify a list of miRNAs predicted to target genes involved in DNA repair, recombination, and replication, chromatin remodeling, cell cycle, and cell death in the model legume *Medicago truncatula*.

Finally, as a follow up of the bioinformatic study by Bellato et al., the research work of Gualtieri et al. provides experimental evidence on the involvement of selected miRNAs in DDR-associated pathways. The authors have developed an experimental system based on the use of specific chemical agents (camptothecin and NSC120686) know to inhibit the activity of topoisomerase 1 and tyrosyl-DNA phosphodiesterase 1 enzymes. These chemical agents were found to affect the DDR as evidenced by the accumulation of DNA damage and cell death combined with altered transcription profiles of key DDR players in DNA repair and cell cycle regulation. The expression of miRNA-target gene pairs was investigated evidencing that when a miRNA is upregulated, its predicted DDR-target gene is downregulated. The contrasting expression profiles observed support the evidence that these miRNAs (miR156a, miR172c-5p, miR2600e, miR395e, and miR5741a) could repress the expression of these targets (*UBE2A*, *RAD54-like*, *5AT*, *DMAP1*, and *E2FE-like*).

Overall, this collection of articles attentively underlines the gaps-of-knowledge existing with regards to the miRNA-mediated regulation of DDR in plants, while encouraging further research still needed to shed light on this complex topic.

AUTHOR CONTRIBUTIONS

All authors equally contributed to write the Editorial and discuss about the articles published in the Research Topic.

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REVIEW
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Redox Balance-DDR-miRNA Triangle: Relevance in Genome Stability and Stress Responses in Plants

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Plants are continuously faced with complex environmental conditions which can affect the oxidative metabolism and photosynthetic efficiency, thus leading to the over-production of reactive oxygen species (ROS). Over a certain threshold, ROS can damage DNA. DNA damage, unless repaired, can affect genome stability, thus interfering with cell survival and severely reducing crop productivity. A complex network of pathways involved in DNA damage response (DDR) needs to be activated in order to maintain genome integrity. The expression of specific genes belonging to these pathways can be used as indicators of oxidative DNA damage and effective DNA repair in plants subjected to stress conditions. Managing ROS levels by modulating their production and scavenging systems shifts the role of these compounds from toxic molecules to key messengers involved in plant tolerance acquisition. Oxidative and anti-oxidative signals normally move among the different cell compartments, including the nucleus, cytosol, and organelles. Nuclei are dynamically equipped with different redox systems, such as glutathione (GSH), thiol reductases, and redox regulated transcription factors (TFs). The nuclear redox network participates in the regulation of the DNA metabolism, in terms of transcriptional events, replication, and repair mechanisms. This mainly occurs through redox-dependent regulatory mechanisms comprising redox buffering and post-translational modifications, such as the thiol-disulphide switch, glutathionylation, and S-nitrosylation. The regulatory role of microRNAs (miRNAs) is also emerging for the maintenance of genome stability and the modulation of antioxidative machinery under adverse environmental conditions. In fact, redox systems and DDR pathways can be controlled at a post-transcriptional level by miRNAs. This review reports on the interconnections between the DDR pathways and redox balancing systems. It presents a new dynamic picture by taking into account the shared regulatory mechanism mediated by miRNAs in plant defense responses to stress.

Keywords: redox balance, DDR, miRNA, redox-sensitive TFs, cell cycle checkpoints

INTRODUCTION

The maintenance of the cellular redox balance is a major biological attribute influencing growth, development and survival in plant and animal systems (de Pinto et al., 1999, 2015; Pellny et al., 2009; Chiu and Dawes, 2012). In animal systems, a mild oxidative environment has been observed to activate a signaling pathway leading to cell proliferation (Menon et al., 2003; Menon and Goswami, 2007). Interaction between the epidermal growth factor and their specific receptor stimulates cell proliferation by the generation of a low amount of reactive oxygen species (ROS; Menon and Goswami, 2007). In plants, a strong correlation between the cellular redox state and cell cycle block has been clearly observed in the root quiescent center, a group of spatially defined cells that are blocked in G0 (Jiang and Feldman, 2005; Jiang et al., 2006; Dinneny et al., 2008). An increase in ROS production generally causes a cell cycle arrest before the activation of the cell death program (Chiu and Dawes, 2012; de Pinto et al., 2012). As a common feature of eukaryotic organisms, it has been hypothesized that cell cycle progression is driven by an intrinsic redox cycle consisting in regulated reductive and oxidative phases (Chiu and Dawes, 2012). Glutathione (GSH), the most abundant non-protein thiol in the cell, seems to be a major actor in the redox fluctuations normally occurring during cell proliferation in animal and plant cells (García-Giménez et al., 2013). Alterations in the cell redox potential may also be responsible for the abnormal proliferation of cancer cells which have a "constitutive" decrease in the cellular redox potential, and therapies able to adjust their cellular redox balance have been proposed (Hoffman et al., 2001, 2008). In plants, phythogen toxins blocking cell proliferation induces an alteration in GSH fluxes between nucleous and cytosol (Locato et al., 2015). Thus, sensing the redox state at tissue, cellular and subcellular levels is needed to accurately allow cell cycle progression in the right redox environmental conditions, linking the cell stress response to the cell cycle checkpoint pathway (Pearce and Humphrey, 2001).

The maintenance of the cellular redox balance is also a crucial attribute influencing plant development. Plant embryogenesis has indeed been correlated to a shift in the cell environment toward a more oxidized state (Belmonte and Stasolla, 2007; Stasolla et al., 2008; Becker et al., 2014) and the circadian clock also seems to be regulated at the redox level and vice versa (Lai et al., 2012). Moreover, the cell redox state is intrinsically correlated to the cell metabolic status and consequently it is presumed to be tightly linked to cell energy efficiency. In aerobic organisms, perturbations in the cell redox status are reflected in metabolic efficiency, calculated as the ratio between oxygen consumption and ATP production (Giancaspero et al., 2009). In line with this, in plants, environmental stressing conditions that perturb the cellular redox status have been found to impair the mitochondrial metabolism (Vacca et al., 2004; Valenti et al., 2007). Thus, metabolic efficiency can be monitored by assaying the mitochondrial respiration pathway. In the yeast model, the metabolic cycle, which consists of respiratory (oxidative phase) and fermentative/non-respiratory (reductive phase) phases, seems to be synchronized to cell cycle progression, with mitosis and DNA replication occurring during the reductive phase and G1 during the oxidative phase (Tu et al., 2005). This synchronization may act as a protective mechanism toward genome integrity, thus enabling DNA synthesis to occur in a non-oxidative environment (Chen et al., 2007).

Plant exposure to stressful conditions, both exogenous (solar UV radiation, high soil salinity, drought, chilling injury, air and soil pollutants including heavy metals) and endogenous (metabolic by-products) in nature, can compromise genome integrity. Due to their sessile lifestyle, and the presence, for all the lifespan, of a small population of the same meristematic cells continuously dividing for allowing organism growth, plants have evolved various strategies to cope with environmental constraint conditions (Spampinato, 2017). Among these, the continuous exposure to sunlight represents a dramatic challenge to genome integrity and to genome transmission to the subsequent generation (Roy, 2014). The DDR specifically aims to aid plants to cope with the detrimental effects of genotoxic stress. DDR is a complex signal transduction pathway, which detects DNA damage signals and transduces those signals to execute cellular responses. Both redox systems and DDR pathways are usually tightly regulated through the coordinated activities of cellular oxidants/antioxidants and DNA damage/repair-signaling pathways (**Figure 1**). It is well-known that intracellular ROS acts both as a cellular damaging compound and as a signaling molecule, all depending on its concentration and localization (Foyer and Noctor, 2003; Jeevan Kumar et al., 2015; Mittler, 2017). Links between ROS and DDR pathways have been hypothesized but not yet clearly demonstrated. For instance, studies on animal cells treated with neocarzinostatin (a radiomimetic that causes the formation of double-strand breaks) have shown that ROS induction is partly mediated by increasing levels of histone H2AX, a biomarker for DDR (Kang et al., 2012). Hence, ROS generate DNA damage while being regulated by the DNA damage-signaling pathways.

All the evidence outlined above makes controlling the cell redox balance a major regulator of virtually all plant metabolic re-arrangements occurring in growth, development and defense strategies. Regulation of all the metabolic transitions experienced by DNA (first and foremost, transcription, replication, and repair) within the cells is expected to be tightly connected with redox signaling pathways. Furthermore, the maintenance of cellular redox homeostasis and genomic integrity can be modulated by the activity of microRNAs (**Figure 1**; miRNAs). This review reports on the influence of various redox active systems on DNA damage response pathways and plant transcriptome as well as on post-transcriptional gene expression regulation mediated by miRNA.

DDR AS A KEEPER OF GENOME STABILITY

DNA damage response is an evolutionary conserved, complex network that includes signal transduction pathways composed of sensors, transducers, mediators, and effectors, dedicated to safeguard genome integrity. Several comparative studies have

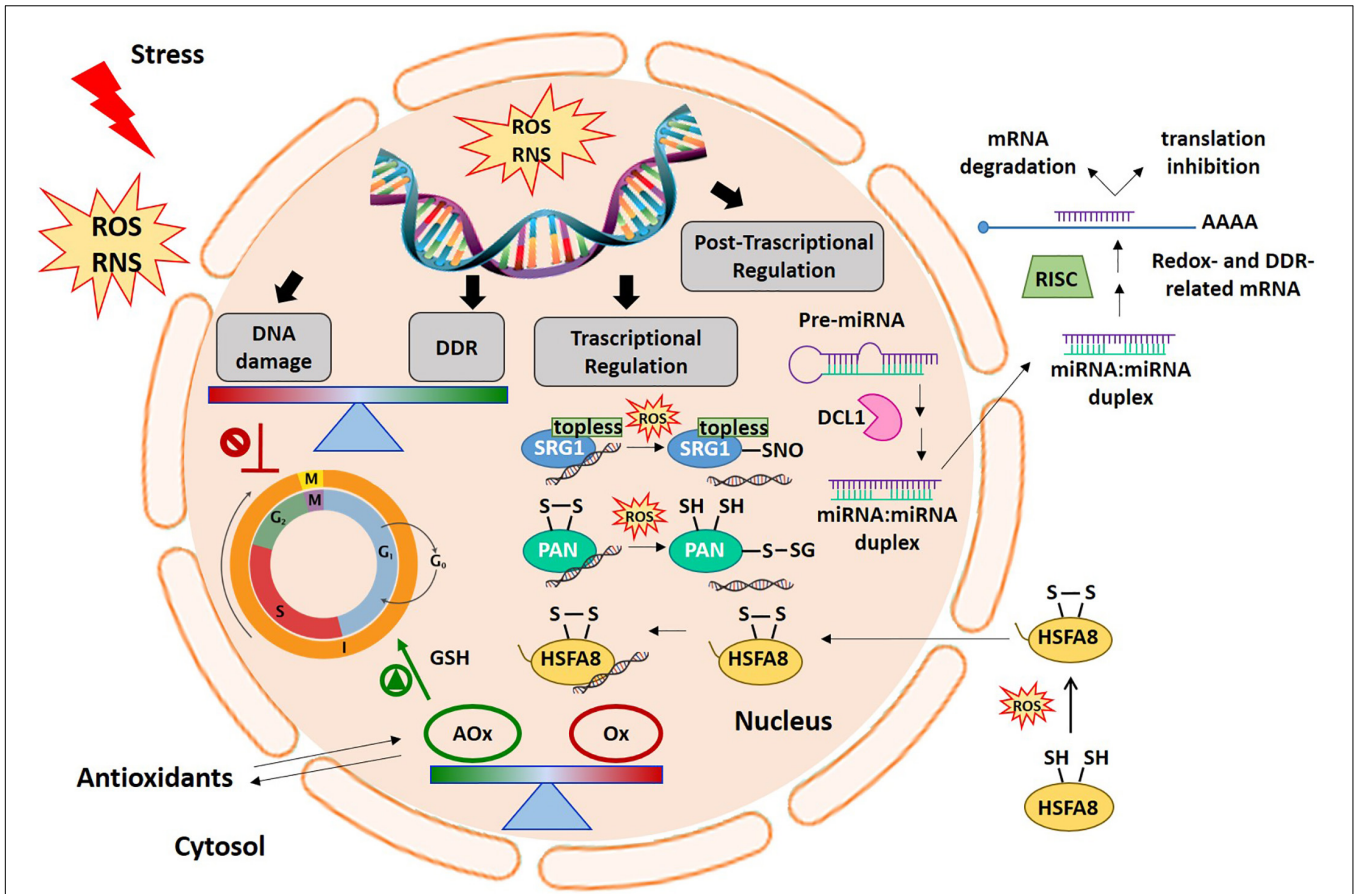

FIGURE 1 | Cellular redox balance-DDR-miRNA triangle. An increase in ROS production generally occurs early under different stress conditions. ROS and redox signals move through different cell compartments. In the nucleus, ROS accumulation can cause DNA damage thus inducing cell cycle arrest. The DDR specifically aims to help plants cope with the negative effects of genotoxic stress. Alterations in antioxidant and oxidant balance in the nucleus are required to promote cell cycle progression in the right redox environment. In this context, ROS and redox signals are involved in the regulation of gene expression at transcriptional and post-transcriptional levels. The picture shows some examples of the redox dependent transcriptional mechanisms involving some redox-sensitive TFs, such as SRG1, PAN, and HSFA8, in gene expression regulation. At the post-transcriptional level, the figure also shows the modulation of redox- and DDR-related target mRNAs by miRNAs. AOx, antioxidants; DCL1, DICER-like1; DDR, DNA damage responses; HSFA8, heat shock factor A8; miRNA, microRNA; Ox, oxidants; PAN, PERIANTHIA; RNS, reactive nitrogen species; ROS, reactive oxygen species; SRG1, SNO-regulated gene 1.

highlighted the conserved features of the core DDR machinery across eukaryotes, including plants and mammals, as well as the presence of unique characteristics in plants (DiRuggiero et al., 1999; Singh et al., 2010; Spampinato, 2017; Nikitaki et al., 2018). Most DDR components are ancestral genes that appeared early in the phylogenetic tree and subsequently expanded and shaped throughout evolution. Based on the detection of a DNA lesion by dedicated sensors, various pathways may be triggered, leading to the activation of cell cycle checkpoints, DNA repair, or programmed cell death (PCD). Endoreduplication, consisting of DNA replication in the absence of cytokinesis, represents a plant-distinctive process, which is also part of DDR (Yoshiyama et al., 2013b).

Most of the knowledge regarding DDR and DNA repair pathways, gained through decades of studies on yeast, bacteria, and mammals, has highlighted its function in plant biology (Spampinato, 2017). Indeed, functional and/or structural homologs of various DDR factors found in animals have been identified in model plants such as Arabidopsis (Spampinato, 2017) and *Medicago truncatula* (Balestrazzi et al., 2011). Some exhaustive examples are: MRE11 (Meiotic Recombination 11), RAD51, NBS1 (Nijmegen breakage syndrome 1) proteins, constituting the MRN complex, and RPA (Replication protein A). The MRN complex is required for double-strand break (DSB) recognition in the DDR pathway involving ATM (Ataxia Telangiectasia Mutated) kinase (Yoshiyama et al., 2013a), while RPA binds to single-stranded DNA (ssDNA) lesions associated with DNA replication in a pathway involving the ATR (ATM and Rad3-related) protein. The ATM and ATR transducers amplify and transduce signals to subsequent effectors through a phosphorylation-mediated cascade of events resulting in the activation of downstream processes (cell cycle arrest with the critical choice between DNA repair and PCD) (Culligan et al., 2004, 2006; Yoshiyama et al., 2013a,b). For instance, ATM and ATR transducers induce phosphorylation of the histone-variant H2AX (Dickey et al., 2009; Yuan et al., 2010)

which, in the γH2AX phosphorylated form, acts as a DNA damage signal and recruits several proteins to the DSB site (Petrini and Stracker, 2003; Yoshiyama et al., 2013a). In yeast and mammals, after ATR activation, serine/threonine-protein kinases CHK1 (checkpoint kinase) and CHK2 were phosphorylated by ATR and ATM, respectively, with a consequent activation of cell-cycle checkpoints (Bartek et al., 2001; Chen and Sanchez, 2004). Arabidopsis appears to have no CHK1 and CHK2 orthologs. Considering that some of the substrates of CHK1 and CHK2 in animals, such as the mediator BRCA1 (breast cancer susceptibility gene 1), and E2F1 (E2F Transcription Factor 1), are also present in plants (Lafarge and Montané, 2003; Inze and de Veylder, 2006), it has been suggested that other kinases may work as functional homologs of CHK1 and CHK2 (Yoshiyama et al., 2013b). Studies on the Arabidopsis *atm* and *atr* mutants have shown that in addition to the conserved function in DDR, ATM and ATR play a different role in the life of plants (Garcia et al., 2003; Culligan et al., 2004). Yan et al. (2013) reported an intriguing finding linking the plant immune system to DNA damage. They demonstrated that the plant hormone SA induces DNA damage in the absence of a genotoxic agent, and the DDR components, ATR and RAD17 (radiation sensitive) are required for adequate plant immune responses, thus suggesting the role of DDR in the defense against pathogens. In contrast, Rodriguez et al. (2018) reported DNA damage as a consequence of autoimmune response rather than actively produced host-DNA damage aimed at stimulating resistance to pathogens.

The various factors involved in DDR are temporally and spatially regulated and activated through the action of mediators that recruit additional substrates and control their association with damaged DNA (Stewart et al., 2003; Stracker et al., 2009). Several mediators are known in human cells, such as MDC1 (mediator of DNA-damage checkpoint protein 1), 53BP1 (p53-binding protein), BRCA1 (Breast cancer susceptibility 1), related to the ATM pathway, TOPBP1 (topoisomerase 2-binding protein 1), and CLSPN (Claspin), involved in the co-regulation of the ATR pathway. Plants lack counterparts for some DDR mediators (e.g., MDC1 and 53BP1) (Yoshiyama et al., 2013b; Nikitaki et al., 2018). However, there are DDR components exclusively found in plants, such as SMR (Siamese-related) cyclin-dependent protein kinase inhibitors, some chromatin remodelers (CHR complexes), and several DNA and histone methyltransferases such as CMT3 [DNA (cytosine-5)-methyltransferase 3], SDG26 (SET domain group 26), SUVH5 (histone-lysine N-methyltransferase, H3 lysine-9 specific) (Nikitaki et al., 2018). Interestingly, the p53 effector, which is a TF acting as tumor suppressor in animal cells, does not exist in plants. In animals, the master regulator p53 rules the fate of the cell following DNA damage, which triggers cell-cycle arrest and then DNA repair or apoptosis (Helton and Chen, 2007). A similar role in plants has been ascribed to the TF SOG1 (suppressor of gamma response 1), a component of the NAC (NAM-ATAF1/2-CUC2) family (Preuss and Britt, 2003; Yoshiyama et al., 2009). SOG1 regulates more than 100 genes and similarly to p53, induces several pathways including cell cycle arrest, DNA repair, PCD, and endoreduplication (Yoshiyama et al., 2013b; Yoshiyama, 2015). It is thus clear that most of the DDR factors are well preserved in animals and plants, although various key components are unique to plants.

DNA Damage Repair Mechanisms Activated by DDR Pathways

Of the pathways triggered by DDR effectors, DNA repair mechanisms are crucial in maintaining genome integrity. Several pathways are involved in the correction of various types of DNA lesions including: (1) direct repair (DR) or photoreactivation (Jiang et al., 1997), (2) mismatch repair (MMR), (3) base- and nucleotide excision repair (BER, NER) (Shuck et al., 2008; Peña-Diaz and Jiricny, 2012; Jiricny, 2013), (4) double-strand break repair (DSBR), which includes non-homologous end joining (NHEJ), and homologous recombination (HR) mechanisms (Puchta and Hohn, 1996).

The DR is a light-dependent pathway that relies on the activity of flavoenzymes, called photolyases, carrying the two electron-reduced forms of FAD (FADH) as photocatalysts (Sancar, 2003). After binding to the DNA lesion, the enzymes remove the damage following absorption of blue light in the 300–600 nm range (Tuteja et al., 2009). The activity of photolyases is specific to plants, since it seems to be absent in humans and other placental animals (Essen and Klar, 2006). On the other side, MMR is present in all organisms, and corrects replication and genetic recombination errors, which result in poorly matched nucleotides. In eukaryotes, the lesion detected by MutS homolog (MSH) proteins is repaired through enzymatic complexes operating an endonucleolytic cut on the neo-synthetized strand, thus restoring the correct sequence through the action of specific DNA polymerases (Marti et al., 2002; Spampinato, 2017).

The BER mechanism is responsible for the repair of damaged single bases resulting from deamination, alkylation, oxidized bases, abasic (apurinic and/or apyrimidinic, AP) sites, and single-strand breaks (SSBs) (Tuteja et al., 2009). It consists of the excision of the damaged base by a DNA glycosylase followed by the consecutive action of at least three enzymes, an AP endonuclease, a DNA polymerase, and a DNA ligase (Stivers and Jiang, 2003). The 8-oxoguanine DNA glycosylase (OGG1), uracil DNA glycosylase (UNG), and formamidopyrimidine DNA glycosylase (FPG), are some examples of plant DNA glycosylases with roles in stress responses (Gutman and Niyogi, 2009; Macovei et al., 2011). Aside being extensively studied in model plants, the pathway has also been characterized in potato mitochondria where it is mostly involved in the repair of DNA damage related to ROS production (Ferrando et al., 2018). While BER removes small DNA lesions, the NER pathway repairs the main DNA lesions causing extensive distortion in the double helix, such as UV-products and bulky covalent adducts (Kunz et al., 2005). The NER mechanism has mostly been studied in Arabidopsis and rice and investigations on NER genes have also been conducted in other plants such as poplar and sorghum (Singh et al., 2010; Spampinato, 2017). Proteins belonging to the RAD family are involved in DNA lesion recognition in NER. For instance, the RAD23 family of genes has been well characterized in Arabidopsis by developing multiple mutant

plants. The triple and quadruple mutants for *rad23a*, *rad23b*, *rad23c*, and *rad23d* genes have shown clear phenotypic changes resulting in dwarfed plants or reproductive lethally mutants. However, single and double mutants have not shown evident differences, thus suggesting a mostly overlapping function of the four genes.

Repairing the DSBs is mostly carried out by DBSR systems. Studies on DBSR mechanisms have been increasing, not only because of their importance in DNA repair but also as tools to modify plant genomes (Baltes and Voytas, 2015; Sprink et al., 2015). DBSR mechanisms mainly include the HR and NHEJ pathways. HR occurs only when two DNA duplexes contain extensive homology regions, while NHEJ enables DSBs to be repaired in the absence of sequence homology. Given the requirement of a sister chromatid as a template, HR is restricted to the S and G_2 phases of the cell cycle, while NHEJ is active throughout the cell cycle and does not rely on a template (Brandsma and Gent, 2012). The error-free HR pathway uses several enzymes including the ssDNA-binding protein RAD51 recombinase (Chapman et al., 2012; Spampinato, 2017). The balance between the HR and NHEJ pathways is essential for genome stability. Besides the well−characterized Ku−dependent NHEJ pathway (classical non-homologous end-joining, C-NHEJ), an XRCC1(X-ray cross-complementation group)-dependent pathway (alternative non-homologous end-joining, A-NHEJ) has been observed both in humans and in plants (Decottignies, 2013; Bétermier et al., 2014; Williams et al., 2014; Sfeir and Symington, 2015; Spampinato, 2017). C-NHEJ is dominant in the G_1 and G_2 phases of the cell cycle, while A-NHEJ preferentially acts in the S-phase (Karanam et al., 2012; Truong et al., 2013). A-NHEJ takes place in the absence of key C-NHEJ factors, and requires the alignment of microhomologous sequences. The pathway is thus also referred to as microhomology-mediated end-joining (MMEJ). Unlike HR, the lack of a homology sequence in NHEJ leads to an error-prone type of repair, frequently resulting in small insertions, deletions, or substitutions at the break site (Chapman et al., 2012).

DDR in Relation to Redox-Based Mechanisms

Redox-based mechanisms would seem to play a key role in the modulation of DNA damage sensing, signaling, and repair. Although there is extensive knowledge in animal systems (Kim et al., 2013; Mikhed et al., 2015; Somyajit et al., 2017), there are few reports on redox signaling and redox-mediated control of DNA repair in plants (Zhang, 2015). Due to the complexity of such molecular networks and in an attempt to draw a representative picture of the state of the art in the plant kingdom, attention has focused on specific players that have been identified at the crossroads of the redox and DDR pathways.

One case relates to Fe-containing proteins (e.g., Fe–S cluster proteins and hemoproteins) which use Fe as a cofactor and play critical roles in several aspects of genome maintenance, including telomere maintenance and cell cycle control in both animals and plants (Zhang, 2014, 2015). In Arabidopsis, several Fe-containing proteins with key functions in genome stability, including DNA helicases and DNA glycosylases, have been characterized. For example, RAD3 (also known as UVH6), the plant homolog of the human XPD and yeast RAD3 proteins, is an essential helicase required for NER function (Liu et al., 2003). Among the known 26 DNA glycosylases, only DEM (DNA glycosylase DEMETER), DML1 (DEMETER-like 1, AtROS1), DML2, and DML3 proteins contain a Fe-S cluster and participate in DNA methylation (Ortega-Galisteo et al., 2008). The biogenesis of Fe-S proteins requires dedicated cluster assembly pathways (Lill and Mühlenhoff, 2008). The highly conserved cytosolic Fe-S cluster assembly (CIA) machinery is required for the transfer of these clusters to target proteins, including those involved in genome maintenance, and impairment of the CIA pathway possibly compromises genome integrity (Netz et al., 2014). Other pathways are located within subcellular compartments such as ISC (iron–sulfur cluster) in mitochondria and SUF (sulfur mobilization) in plastids (Couturier et al., 2013). Mutations that target genes coding for the CIA subunit, including AE7 (AS1/2 enhancer 7) and ATM3 (ABC transporter of the mitochondrion 3), result in DNA damage accumulation and enhanced HR rates (Luo et al., 2012). Seedlings of the Arabidopsis *ae7* mutant have shown increased sensitivity to the DNA damage agents, methylmethane sulphonate and cisplatin. The *ae7* mutant cells have also been shown to be blocked at the G2/M transition of the cell cycle and revealed increased expression of DDR genes, including PARP (Poly(ADP-ribose) polymerase), BRCA1, GR1 (Gamma response 1), and TOS2 (Ribonucleotide reductase-like catalytic subunit), involved in DSB repair and genome maintenance (Luo et al., 2012). The defective CIA pathway would seem to cause genotoxic damage, which triggers cell cycle arrest and DDR. Similarly, increased sensitivity to genotoxic agents and up-regulation of DDR genes have been observed in the Arabidopsis *atm3* mutant lacking the ATM3 function (Luo et al., 2012).

Chromatin remodeling is also a key aspect since it is necessary for the access of the DDR protein to the damaged DNA site. Evidence of the redox-mediated modulation of chromatin remodelers has been provided in animal systems. Duquette et al. (2018) reported that lysine demethylase 1 (LSD1/KDM1A), a flavin adenine dinucleotide (FAD)-dependent amine oxidase able to demethylate the lysine 4 residue of histone H3, triggers H_2O_2 accumulation as a by-product of its chromatin remodeling activity during the early steps of DDR. This is the first evidence that ROS can be generated *ex novo* in human cells as part of DDR, at a specific damaged site. In addition, the local production of H_2O_2 can control the activity of DNA repair enzymes recruited at the lesion. This suggests that the local redox environment might modulate the two major DBS repair pathways, namely HR and NHEJ (Duquette et al., 2018). It is possible that a similar mechanism also takes place in plant cells. The Arabidopsis genome encodes four LSD1 homologs named LSD1-like (LSDL), of which LSDL1 and LSDL2 control histone H3 methylation only around and within the heterochromatin region containing the floral repressors FLC (FLOWERING LOCUS) and FWA, which is crucial for the timing of the developmental transition to flowering (Jiang et al., 2007). Unlike

for animals, there is currently no evidence of the role of plant LSD1-like proteins in DDR.

In the complex and variegated scenario of intersecting DDR and redox mechanisms, it is also possible that the same protein fulfills a dual role, acting in a redox context as well as maintaining genome stability. In the PARP-like genes, found in eukaryotes, the PARP catalytic domain is associated with other functional domains (Vainonen et al., 2016). The Arabidopsis protein RCD1 (inactive poly [ADP-ribose] polymerase) contains a WWE domain (Trp-Trp-Glu, involved in protein-protein interactions occurring in ubiquitination and ADP ribosylation) (Aravind, 2001) and an RST (RCD-SRO-TAF4) domain also responsible for protein-protein interactions. Proteins that contain this domain combination, specific to plants, are named SIMILAR TO RCD-ONE (SRO) (Jaspers et al., 2010). According to Liu et al. (2014), overexpression of the *TaSRO* gene in Arabidopsis provides increased tolerance to genotoxic stress induced by UV irradiation and H_2O_2 treatments. The authors ascribed genome integrity to the enhanced PARP activity detected in the *TaSRO*-overexpressing cells that positively affected DDR, resulting in higher levels of the ATM ROS sensor. Interestingly, the *TaSRO*-overexpressing cells accumulated more ROS than the control lines, under both non-stressed and stressed conditions, combined with an efficient antioxidant response that ensured redox homeostasis (Liu et al., 2014). Thus, the particular structural features of *TaSRO* enable this protein to play a dual role in the stress response, acting through the modulation of redox parameters and genome maintenance.

Arabidopsis *apx1/cat2* double mutants that constitutively activate DDR at a transcriptional level represent an interesting example of redox-DDR interaction (Vanderauwera et al., 2011). This confers tolerance against various stresses in the double mutants, since the induced DDR is active also in the absence of DNA damage. DDR induction was inhibited under high CO_2 in the double mutants, suggesting that the ROS production derived from photorespiration caused DDR induction at a constitutive level in the double mutants also under standard conditions. In addition, the WEE1 serine/threonine kinase-dependent cell cycle checkpoint was activated in *apx1/cat2* mutants, which suggests that cell cycle arrest is part of the signaling pathway activated by ROS involving DDR induction (Vanderauwera et al., 2011).

ROLE OF REDOX BALANCE IN TRANSCRIPTIONAL CONTROL

Redox Regulated Transcriptome Re-programming

Redox-based mechanisms play a key role in the regulation of gene expression. Several studies based on omics approaches have demonstrated that ROS induce transcriptional modifications by direct or indirect mechanisms. This experimental evidence has been mainly obtained by manipulating cell ROS levels and/or redox balance in pharmacological or genetic contexts. Effective case-by-case studies were obtained by using mutants defective in enzymatic antioxidant systems (such as catalase, ascorbate peroxidases and ascorbate oxidase – AO; Vanderauwera et al., 2005; Gadjev et al., 2006; Pignocchi et al., 2006; Rasool et al., 2017) as well as treatments with ROS-generating systems, with electron transfer inhibitors in chloroplast and mitochondria or oxidative stress triggering agents (Gadjev et al., 2006; Broda and Van Aken, 2018). The transcriptomic changes appear to be finely tuned depending on ROS types and production site within the cell (Locato et al., 2018). In fact, various environmental backgrounds can promote ROS increases, above all in the apoplast, chloroplasts, mitochondria, and peroxisomes. To give some examples: biotic stresses as well as high light (HL), salt, and drought have been related to apoplastic ROS accumulation by the activation of plasma membrane-located NADPH oxidases belonging to the family of respiratory burst oxidase homolog proteins (RBOH; Ma et al., 2012; Kadota et al., 2015; Kurusu et al., 2015; Li et al., 2015; Evans et al., 2016; He et al., 2017; Karpinska et al., 2018); HL also induces chloroplast ROS production (Foyer and Shigeoka, 2011), whereas photorespiration mainly causes ROS production in the peroxisomes (Foyer et al., 2009), and a number of abiotic stresses increase ROS production in mitochondria (Foyer and Noctor, 2003). Controlled fluxes of redox active molecules (oxidants and antioxidants) between organelles and cytosol, regulate redox mechanisms which, in turn, results in the control of gene expression within the nuclei (Locato et al., 2018). This gene expression reprogramming possibly enable plants to by-pass a stressful situation or a metabolic impairment. Sewelam et al. (2014) demonstrated that hydrogen peroxide (H_2O_2) production triggered by the activation of photorespiratory pathway induced a different set of nuclear genes depending on the ROS production site. Their study used Arabidopsis plants overexpressing glycolate oxidase 5 (GO5), producing ROS in the chloroplast under photorespiratory conditions, and a catalase defective line (*cat2-2*), where ROS over-production occurred in the peroxisomes during photorespiration. When ROS were mainly produced in the chloroplasts, the induced genes mostly belonged to the functional categories of the transcription factors (TFs), proteins involved in signaling and metabolic pathways, and in defense or detoxification. Differently, peroxisome-derived ROS mainly induced the expression of genes involved in protein folding and repair (such as chaperones and heat shock proteins – HSPs), along with defense and detoxification processes. Therefore, different ROS responsive genes were identified to be linked to redox impairment occurring in specific intracellular contexts. A meta-analysis (Vandenbroucke et al., 2008) revealed that yeast, plants, and animals share at least four families of H_2O_2-responsive genes: a class of HSPs, GTP-binding proteins, Ca^{2+}-dependent protein kinases, and ubiquitin-conjugating enzymes. Antioxidant genes shows an H_2O_2-dependent up-regulation only in prokaryotes. This probably depends on the fact that in eukaryotes antioxidant genes show a high constitutive expression probably as an evolutionary acquisition. Thus, in eukaryotes, antioxidant systems are mainly controlled at post-translational level (Vandenbroucke et al., 2008). For example, the synthesis of GSH is controlled by post-translational modification based on a

thiol switch mechanism. Oxidative conditions (also determined by stressful conditions) activate the enzyme that catalyzes the limiting biosynthetic step in GSH production, γ-Glutamyl cysteine synthetase (γ-ECS), by disulphide bond formation in the γ-ECS homodimer. This also represents a controlled redox loop involving GSH (reviewed by Yi et al., 2010).

Glutathione is a major redox soluble metabolite controlling the cell redox balance under physiological and perturbed situations in both developmental and defense contexts (Noctor et al., 2012; Hernández et al., 2017; Locato et al., 2017). The effect of GSH on the transcriptome has been investigated in various studies (Cheng et al., 2015 and references therein; Hacham et al., 2014). GSH feeding of Arabidopsis seedlings appears to induce the expression of stress-related genes and down-regulates developmental correlated genes (Hacham et al., 2014). High GSH levels have also often been correlated to increased stress tolerance (Cao et al., 2017; Ferrer et al., 2018; Formentin et al., 2018). Conversely, GSH deficiency in Arabidopsis root meristem less 1 mutant (*rml1*) has been shown to affect root growth and architecture through a massive transcriptome re-programming. This result has been confirmed in other GSH deficient mutants (*cad2-1*; *pad2-1*; *rax1-1*) presenting different mutations in the GSH1 gene, encoding the GSH biosynthetic enzyme γ-ECS, and in condition of GSH depletion by treatment with buthionine sulfoximide (BSO), an inhibitor of GSH biosynthesis. In all these contexts, GSH deficiency affected above all the expression of those genes involved in cell cycle progression, especially those involved in G2-M transition. On the other hand, the expression of several genes related to redox signaling were less modified probably because the GSH redox state did not change in the mentioned experimental conditions. On the other hand, heat shock (HS) responsive genes were down-regulated, suggesting that the lack of GSH affected redox signaling leading to the expression of these genes. This suggests that GSH is generally required in the induction of oxidative-stress related genes. The redox state of nucleus and cytosol in Arabidopsis root cells has also been monitored in *rml1* mutants and in wild type under BSO treatment. In both compartments, the GSH depletion triggered an increase in the redox state, suggesting that the link between root development, growth, and cell redox state is strongly dependent on the GSH level controlling transcriptome re-programming (Schnaubelt et al., 2015). Karpinska et al. (2017) demonstrated that the nuclear redox state is also prone to oxidation when different plant tissues and cell types were treated with inhibitors of mitochondrial and chloroplast electron transfer, which enable oxidative impair within the cells. The authors observed transcriptome re-programming as a consequence of nucleus oxidation, leading to the retrograde regulation of the expression of genes, mainly related to organelle functions. The GSH-dependent control of the nuclear redox state thus appears to be crucial in interconnected signaling networks which are involved in the organelle cross-talk determinant for gene expression regulation. It has also been demonstrated that an increase in GSH, obtained by exogenous treatment or genetically, enhances the translational efficiency of Arabidopsis plants. This enhancement can be inferred from the changes observed in the polysomal fraction profile, which is indicative of the number of active translation events. An increase in the GSH level seems to activate the translation of pre-existing mRNAs of cluster genes related to hormone biosynthesis, proline biosynthesis, stress response, including TFs involved in defense response, root growth, cell cycle, metabolism, and sulfur assimilation. These data are in accordance with the protective role of GSH supplementation against a plethora of different stress conditions. It also suggests an overall control of the translatome and transcriptome of GSH in plants, probably also correlated to the control played by this metabolite in development and cell proliferation (Cheng et al., 2015; Locato et al., 2015).

Another non-enzymatic antioxidant molecule which intracellular concentration affecting the cellular redox state is the ascorbate (ASC). The ASC level and redox state have been correlated to cell proliferation (de Pinto et al., 1999; Pellny et al., 2009; de Simone et al., 2017; Kka et al., 2018), plant development (Foyer and Noctor, 2009; Paradiso et al., 2012; Cimini et al., 2015) and defense (Kiddle et al., 2003; Sabetta et al., 2019). In fact, ASC treatment of quiescent center cells re-activated the cell division process (Liso et al., 1988). However, according to the literature, the possible involvement of ASC in the control of transcriptional events has not been characterized as well as it has been for GSH. A recent system biology study (Stevens et al., 2018) investigated the effect of ASC metabolism perturbation on the transcriptomes, metabolomes, and proteomes of tomato fruits. The study took into account the RNAi lines for AO, L-Galactono-1,4-γ-lactone dehydrogenase (GLD) and monodehydroascorbate reductase (MDHAR), which are all enzymes involved in the control of ASC levels and the redox state. Although in this study the analysis was carried out on a particular non-photosynthetic tissue and reported no differences in metabolite and protein levels, it did reveal the role of the ASC pool in controlling those core genes involved in ribosome biogenesis, structure, translation, and protein folding (Stevens et al., 2018). Another study performed a comparative analysis of the leaf transcriptome of Arabidopsis mutants which showed reduced levels of GSH (*rml1*), ASC (*vitc1*, *vitc2*) and ROS detoxification in peroxisomes (catalase 2 defective mutant; *cat2*) and chloroplasts (thylacoydal ascorbate peroxidase defective mutant; *tapx*) (Queval and Foyer, 2012). It revealed that both low GSH and ASC caused significant transcriptome reprogramming, although deficiencies in the two antioxidants seemed to affect different sets of genes. Interestingly, there was a 30% overlap among the sets of genes regulated by low antioxidant levels and impairment of ROS detoxification systems; whereas only 10% of the genes regulated by H_2O_2 increases observed in *cat2* and *tapx* mutants overlapped (Queval and Foyer, 2012).

The Role of Redox Sensitive TF Regulation in DNA Transcriptional Control

Reactive oxygen species can regulate gene expression by modulating the activity of numerous TFs. Several redox-dependent mechanisms controlling TF activity have been described in plants, although this is still an under-investigated field. Redox regulation may include conformational changes in

TFs and TF-binding proteins (positive or negative regulators), or an alteration in their intracellular compartmentalization as well as redox-dependent TF proteolysis. **Table 1** summarizes the information related to 12 redox-regulated TFs that directly target several genes involved in plant stress responses. A more detailed description of these TFs and their mechanism of action is provided in the sub-chapters below.

Redox Sensitive TF Belonging to the ERF/AP2 TF Family

Different proteins belonging to the ERF/AP2 TF family undergo redox regulation. Of these TFs, the Redox Responsive Transcription Factor1 (RRTF1) seems to be involved in redox homeostasis under adverse conditions. The RRTF1 transcript levels were shown to be strongly and rapidly increased in response to singlet oxygen and other ROS as well as biotic- and abiotic-induced redox signals such as aphid infection, HL, and salt stress exposure (Matsui et al., 2008; Jaspers and Kangasjärvi, 2010; Heller and Tudzynski, 2011; Jiang et al., 2011). The regulation of the activity of this TF is still not well understood. An increase in RRTF1 expression was found after *Alternaria brassicacea* infection and/or H_2O_2 treatment. In this context, WRKY18/40/60 has been shown to be required for this up-regulation (Matsuo et al., 2015). In particular, a dynamic sub-nuclear re-localization of WRKY40 is induced by abscisic acid (ABA) treatment in a phosphorylation-dependent manner. Once in the nucleus, WRKY40 binds the promoter region of RRTF1 thereby controlling its gene expression (Pandey et al., 2010). RRTF1 binds to GCC-box-like motifs located in the promoter of RRTF1-responsive genes, thereby favoring an increased defense response under constraint conditions (Matsuo et al., 2015).

The Related to Apetala-2 (RAP2) TFs are also one of the main groups of redox regulated proteins belonging to the ERF/AP2 family. The Arabidopsis RAP2.4 TF class consists of eight members characterized by highly conserved DNA-binding domains with overlapping and specific functions. These RAP2.4 proteins constitute a regulative network in which RAP2.4a is the transcriptional activator of chloroplast peroxidase activity. Other RAP2.4 proteins may function as important modulators since an imbalance in the RAP2.4 pattern can, either positively or negatively, affect the expression of target genes by altering the RAP2.4a transcription (Rudnik et al., 2017). The RAP2.4a TF undergoes dimerization under slightly oxidizing conditions and regulates the induction of three chloroplast peroxidases, namely 2-Cys peroxiredoxin A (2CPA), thylakoid and stromal ascorbate peroxidase (tAPX and sAPX), as well as other enzymes involved in redox homeostasis, such as CuZn-superoxide dismutase (SOD; Shaikhali et al., 2008). Under severe oxidative stress, RAP2.4a oligomerizes, thus suppressing its DNA-binding affinity and consequently reducing the expression of target genes (Shaikhali et al., 2008). The interaction of RAP2.4a with RADICAL INDUCED CELL DEATH 1 (RCD1) supports the activation of RAP2.4a transcriptional activity (Hiltscher et al., 2014).

Another member of the ERF/AP2 TF family involved in the regulation of gene expression in a redox dependent manner is RAP2.12. This TF is anchored at the plasma membrane within an Acyl-CoA binding protein 1 and 2 (ACBP1/2) under aerobic conditions (Gibbs et al., 2011). Upon hypoxia, the interaction RAP2.12-ACBP1/2 is suppressed and RAP2.12 is translocated to the nucleus by a mechanism involving an N-terminal cysteine (Cys). Once inside the nucleus, RAP2.12 activates the expression of hypoxia-responsive genes, such as pyruvate decarboxylase 1 (PDC1) and alcohol dehydrogenase 1 (ADH1) (Licausi et al., 2011). After re-oxygenation, RAP2.12 is subjected to a redox-dependent proteolysis via the oxygen-dependent branch of the N-end rule pathway (Licausi et al., 2011; Licausi, 2013; Kosmacz et al., 2015). An oxygen-dependent oxidation of the penultimate Cys residues at the N-terminus of RAP2.12 occurs under normoxia conditions. This reaction, catalyzed by plant Cys oxidases, leads to RAP2.12 destabilization (Weits et al., 2014).

Redox Sensitive TF Belonging to the Zinc Finger TF Family

Proteins belonging to the zinc finger TF (ZF-TFs) family can also be redox-regulated. For example, the ZF-TF SNO-regulated gene1 (SRG1), which has been proposed as a nuclear nitric oxide (NO) sensor (Cui et al., 2018). NO is a reactive signaling molecule that modulates the expression of defense-related genes. In response to pathogen attack, a nitrosative burst occurs leading to transient NO accumulation. Following pathogen recognition and NO accumulation, SRG1 is expressed and binds a repeated sequence $ACTN_6ACT$ or $ACTN_4ACT$ within promoters of genes coding for immune repressors. This ZF-TF contains an EAR domain required for the recruitment of the co-repressor TOPLESS, thus favoring the transcriptional suppression of target immune repressors (**Figure 1**). An additional increase in NO levels induces the S-nitrosylation of SRG1, above all at Cys87. The SRG1 S-nitrosylation relieves DNA binding and transcriptional repression, thus enabling the expression of negative regulators of plant immunity (**Figure 1**). The S-nitrosylation of Cys87, and possibly other Cys residues paired to the ZF motifs, may lead to Zn^{2+} release and to conformational changes responsible for the altered activity of this ZF-TF (Cui et al., 2018).

Another redox regulated ZF-TF is the ZINC FINGER OF ARABIDOPSIS THALIANA 12 (ZAT12) which has been suggested to be involved in the abiotic stress signaling network. Under iron (Fe) deficiency conditions, H_2O_2 content showed a marked increase, which leads to the establishment of oxidizing conditions. H_2O_2 may function as a signaling molecule that induces the transcription of the FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT). The increase in the H_2O_2 content occurs in a FIT-dependent manner. Under prolonged Fe deficiency conditions, H_2O_2 reduces FIT transcription and activates the transcription of its direct binding partner ZAT12 (Le et al., 2016). In Arabidopsis, ZAT12 transcription has been shown to be up-regulated as a consequence of superoxide anion (O_2^-) treatment (Xu et al., 2017). ZAT12 acts as negative regulator of FIT: in the nucleus, ZAT12 engages FIT through its C-terminal EAR motif in a protein complex thereby altering the balance between active and inactive FIT pools. ZAT12 is also required for the up-regulation of other stress-related genes such, as APX1 and BHLH039 TFs (Davletova et al., 2005; Le et al., 2016). ZAT12 has also been found to undergo proteasome-dependent degradation in the presence of high H_2O_2 levels. The

TABLE 1 | List of redox sensitive TFs and their regulatory mechanism.

TF's family	TF	Redox regulatory mechanism	References
ERF/AP2 TFs	RRTF1	Phosphate-dependent nuclear re-localization of WRKY40 that activate RRTF1 gene expression	Jaspers and Kangasjärvi, 2010; Pandey et al., 2010; Heller and Tudzynski, 2011; Jiang et al., 2011; Matsuo et al., 2015
	RAP2.4a	Conformational state: protein homo-dimerization	Shaikhali et al., 2008; Hiltscher et al., 2014
	RAP2.12	Redox control of the interaction with a binding partner and nuclear re-localization	Gibbs et al., 2011; Licausi et al., 2011; Licausi, 2013; Weits et al., 2014; Kosmacz et al., 2015
ZF-TFs	SRG1	Post-translational modification and redox control of the interaction with a co-repressor	Cui et al., 2018
	ZAT12	Gene expression induction and proteolytic degradation depending on ROS intracellular levels	Davletova et al., 2005; Brumbarova et al., 2016; Le et al., 2016; Xu et al., 2017
bZIP-TFs	PAN	Redox-sensitive DNA-binding controlled by disulphide bridge formation and post-translational modification	Li et al., 2009; Gutsche and Zachgo, 2016
	VIP1	Nuclear re-localization dependent on redox-sensitive interaction with a negative regulator	Takeo and Ito, 2017
	TGA1	Redox-dependent conformational change of the co-activator protein NPR1 that allow its nuclear re-localization and interaction with TGA TFs	Tada et al., 2008; Lindermayr et al., 2010; Kneeshaw et al., 2014; Kovacs et al., 2015
NAC TFs	VND7	Post-translational oxidative modification that affect TF's transactivation activity	Kawabe et al., 2018
HSFs	HSFA8	Redox-dependent conformational change required for nuclear re-localization	Giesguth et al., 2015
	HSFA4A		Pérez-Salamó et al., 2014
	HSFA6B		Yoshida et al., 2010; Huang et al., 2016

EAR motif seems to be crucial for this proteasome-targeting (Brumbarova et al., 2016; Le et al., 2016).

Redox Sensitive TF Belonging to the Basic Leucine Zipper TF Family

The basic leucine zipper TF (bZIP-TFs) is another family including TFs that undergo redox control. A representative example of a redox-sensitive TF belonging to this family is the Arabidopsis TF PERIANTHIA (PAN), which regulates flower organ development and, in particular, the formation of floral organ primordia (Running and Meyerowitz, 1996). PAN was found to bind the AAGAAT motif located in the second intron of the floral homeotic protein AGAMOUS (AG) (Maier et al., 2009). The nuclear interaction of PAN with ROXY1, a plant-specific glutaredoxin (GRX), is crucial for petal development in Arabidopsis (Li et al., 2009). PAN strongly interacts with the AAGAAT motif only under reducing conditions, and redox-sensitive DNA-binding is controlled by the activity of five N-terminal cysteines. Under oxidizing conditions, Cys68 and Cys87, two N-terminal cysteines, can form a disulphide bridge which may alter the conformational structure of this TF, thus changing its ability to bind the DNA (Gutsche and Zachgo, 2016; **Figure 1**). PAN also undergoes redox-dependent post-translational modifications. It has been demonstrated that Cys340, located in a putative transactivation domain, can be S-glutathionylated, thus modifying PAN activity (**Figure 1**). The S-glutathionylation of Cys340 does not affect the PAN DNA binding activity, however, it might indicate an additional redox-dependent strategy capable of altering TF activity (Li et al., 2009; Gutsche and Zachgo, 2016).

The VIRE2-interacting protein 1 (VIP1) is a TF belonging to the bZIP-TF family whose redox-sensitive regulatory mechanism depends on a subcellular relocation due to an altered interaction with a negative regulator. Under control conditions, VIP1

has three phosphorylated serine residues in the HXRXXS motif. In a phosphorylated state, VIP1 can interact with 14-3-3 proteins in the cytosol, and this interaction might inhibit VIP1 nuclear import. Mechanical and hypo-osmotic stress exposure caused de-phosphorylation of VIP1, which resulted in a dissociation of 14-3-3 proteins thereby favoring its nuclear location (Takeo and Ito, 2017).

TGACG-sequence-specific protein-binding (TGA) TFs are bZIP-TFs involved in the redox-regulated activation of defense responses triggering plant immunity under pathogen attack. In Arabidopsis, the salicylic acid (SA)-dependent responses, activated upon pathogen infection, are mediated by the redox-regulated nuclear translocation of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and by an altered interaction between NPR1 and TGA1 and TGA2 TFs (Tada et al., 2008; Kneeshaw et al., 2014). NPR1 is a co-activator protein whose status is tightly controlled by redox changes occurring after pathogen infection or SA treatment (Mou et al., 2003). This protein is kept in the cytosol in a disulphide-bound oligomeric homocomplex. A reduction in the disulphide bond in NPR1 was found to occur in response to SA-induced changes in cellular redox status. The consequent monomerization unmasked a nuclear location signal, which enables the protein to relocate into the nucleus. Thioredoxins h5 and h3 (TRXh5 and TRXh3) reduce the disulphide-binding oligomers thereby favoring NPR1 monomerization and its nuclear translocation (Kneeshaw et al., 2014). In the nucleus, NPR1 seems to interact with TGA TFs and this triggers the expression of defense genes, such as pathogenesis-related protein 1 (PR1) (Tada et al., 2008). NO controls the translocation of NPR1 into the nucleus (Tada et al., 2008) and the DNA binding activity of its interactor protein TGA1 (Lindermayr et al., 2010). The oligomer-to-monomer reaction involves transient site-specific S-nitrosylation. The NO donor S-nitrosoglutathione (GSNO) thus promotes the nuclear accumulation of NPR1, PR1 expression induction and increased GSH concentration upon *Pseudomonas* infection. GSH accumulation has been shown to be crucial not only for cellular redox homeostasis but also for SA accumulation and activation of the NPR1-dependent defense response (Kovacs et al., 2015).

Redox Sensitive TF Belonging to the NAC TF Family

A member of the NAC TF family, named VASCULAR-RELATED NAC DOMAIN 7 (VND7), appears to undergo a reversible oxidative modification (Kawabe et al., 2018). VND7 is involved in xylem vessel cell differentiation (Yamaguchi et al., 2008). Kawabe et al. (2018) found that VND7 is S-nitrosylated at Cys264 and Cys320 located in the C-terminal region near the transactivation domains. The increased S-nitrosylation of VND7 suppresses the transactivation activity of VND7. In this context, a critical role is played by GSNO reductase 1 (GSNOR1) which is thought to be responsible for maintaining cellular S-nitrosothiol homeostasis by regulating the equilibrium between S-nitrosylated proteins and GSNO. The phenotypic traits of the recessive mutant suppressor of the ectopic vessel cell differentiation induced by VND7 (*seiv1*), i.e., an inhibited xylem cell differentiation, have thus been attributed to a loss of function mutation in gsnor1. Consequently, cellular redox state perception by GSNOR1 seems to be important for cell differentiation in Arabidopsis by regulating the post-translational oxidative modification of the TF VND7 (Kawabe et al., 2018).

Redox Sensitive TF Belonging to the HSF, WYRKY, and MYB TF Families

Typical redox sensitive TFs may also be recruited in response to specific adverse environmental situations, for example heat shock factors (HSFs) which activate protective genes in plants subjected to high temperatures or other stress conditions. HSFs recognize the heat stress elements (HSEs) located in promoters of heat-induced targets. Plants have numerous classes of HSFs that are encoded by 21 genes in Arabidopsis (Scharf et al., 2012). HSFs remain inactive in the cytosol by interaction with HSPs. This interaction masks the nuclear location signal and the oligomerisation domain. Under stress conditions, HSPs act as molecular chaperones and HSFs oligomerize and are translocated into the nucleus where they modulate the expression of target genes (Hahn et al., 2011; Mittler et al., 2012; Scharf et al., 2012). A redox-dependent translocation of HSFA8 from the cytosol to the nucleus has been described in Arabidopsis plants subjected to H_2O_2 treatment (Giesguth et al., 2015). Two Cys residues act as redox sensors in AtHSFA8: Cys24, which is located in the DNA binding domain, and Cys269, which is located in the C-terminal part of the protein. Disulphide bond formation between Cys24 and Cys269 may cause a drastic conformational change and induce AtHSFA8 translocation into the nucleus probably by its release from multi-heteromeric complexes (**Figure 1**). In single mutants (AtHSFA8-C24S and AtHSFA8-C269S) and in the double mutant (AtHSFA8 C24/269S), HSFA8 nuclear translocation is thus suppressed under oxidative stress (Giesguth et al., 2015). Similarly, Arabidopsis HSFA4A, described as an H_2O_2 sensor, has been reported to form homodimers (or homotrimers). This mechanism is thought to be required for the transactivation activity of this TF (Pérez-Salamó et al., 2014). HSFA4A expression is enhanced by numerous adverse conditions known to induce ROS accumulation such as salt, paraquat, heat/cold treatment, drought, hypoxia and several pathogens (Sun et al., 2001; Libault et al., 2007; Peng et al., 2012). HSFA4A, in turn, seems to modulate transcriptional activation of a set of target genes involved in mounting defense responses to abiotic and biotic stress conditions, such as APX, HSP17.6A, ZAT6, ZAT12, CTP1, WRKY30, and CRK13. In Arabidopsis-related species, the formation of redox-sensitive disulphide bonds of Cys residues may be a requirement for HSFA4A homodimerization. In addition, Ser309, located between two activator domains, has been identified as the preferential phosphorylation site catalyzed by MPK3 and MPK6 (Pérez-Salamó et al., 2014).

HSFA6B is another redox-regulated HSF which might play a role in the ABA-dependent pathway under salt and dehydration (Yoshida et al., 2010; Huang et al., 2016). HSFA6B is a protein located in both the cytosol and nucleus under normal growth conditions. After ABA or leptomycin treatment, there is an increase in its nuclear location. In the nucleus, HSFA6B may interact with other HSF proteins such as HSFA1A, HSFA1B,

and HSFA2, thereby forming hetero-oligomeric complexes and significantly activating the transcriptional activity of defense genes such as HSP18.1-CI, DREB2A, and APX2 (Huang et al., 2016). HSFA6B seems to have a functional redundancy with the HSFA6A protein during salt and drought stresses. HSFA6A is present at the nucleus and cytosol simultaneously under physiological conditions. However, after salt and drought treatment, HSFA6A has been mainly detected in the nucleus. HSFA6A functions as a transcriptional activator of target genes involved in the enhancement of stress tolerance by its C-terminal moiety. This TF is, in turn, transcriptionally activated by various TFs such as ABF/AREB proteins, MYB96, MYB2, MYC2, and WRKY TFs under salt and drought stress in Arabidopsis (Abe et al., 2003; Seo et al., 2009; Niu et al., 2012; Hwang et al., 2014). In addition, the VOZ1 protein may interact with the DNA-binding domain of HSFA6A under normal growth conditions; however, under high salinity conditions, VOZ1 expression slightly decreases together with its protein content. Thus, freed from interaction with VOZ1, HSFA6A protein can function as a positive regulator of the gene expression involved in tolerance acquisition (Hwang et al., 2014).

A core set of ROS-responsive transcripts has been identified in the systemic acquired acclimation response of Arabidopsis following HL application. Four different TFs, namely GATA8, WRKY48, WRKY53, and MYB30, were found to control HL-dependent transcriptome re-programming. The expression of these TFs peaked 2 min after HL exposure both in local and systemic leaves. They were found to be associated with ROS/Ca^{2+} waves generated under these stress conditions (Zandalinas et al., 2019). MYB30 also regulates oxidative and heat stress responses by modulating cytosolic Ca^{2+} levels in response to H_2O_2 variations through annexin expression modulation. During ROS/Ca^{2+} wave propagation, MYB30 binds the promoters of ANN1 and ANN4, and represses their expression thereby regulating cytosolic Ca^{2+} levels (Liao et al., 2017). WRKY48 and WRKY57 are involved in pathogen- and drought- induced defense responses (Xing et al., 2008; Van Eck et al., 2014; Sun and Yu, 2015) and GATA8 acts as a positive regulator of Arabidopsis seed germination (Liu et al., 2005).

The examples discussed above suggest that under biotic and abiotic stress conditions, ROS cause drastic changes in nuclear gene expression by altering the activity of specific TFs that regulate the synthesis of proteins related to plant stress adaptation.

THE IMPLICATION OF MIRNAS IN REDOX- AND DDR-ASSOCIATED PATHWAYS

Gene expression can be modulated also at post-transcriptional level. At this regard, miRNAs, a type of short non-coding RNAs, have been indicated as promising candidates in the precise regulation of genes by targeting messenger RNAs (mRNAs) for cleavage or directing translational inhibition. miRNAs are generally produced from a primary miRNA transcript, the pri-miRNA, through the activity of nuclear RNase DICER-LIKE 1 (DCL1), while mature miRNAs are incorporated into a protein complex named RISC (RNA-induced silencing complex) (**Figure 1**; Reinhart et al., 2002; Bartel, 2004).

In human cells, recent studies have investigated the interactions between DDR components, redox signaling pathways, and miRNAs (for reviews see Hu and Gatti, 2011; Wan et al., 2014; Bu et al., 2017). An interplay between miRNAs, DDR and redox signaling pathways is possible. Indeed, both DDR and redox signaling can modulate miRNA expression, while miRNAs can directly or indirectly modulate the expression of proteins that are part of DDR and redox signaling. Understanding the roles of miRNAs in DDR and redox signaling along with their implications in complex diseases such as cancer (He et al., 2016; Arjumand et al., 2018), or throughout the aging process (Bu et al., 2016, 2017), are viewed as diagnostic tools or alternative therapeutic treatments (Huang et al., 2013; Badiola et al., 2015).

The situation is quite different in plants, where only very few studies have started to address this complex picture. Yet, miRNAs have been extensively studied in terms of stress responses and exhaustive reviews regarding this aspect are available (Noman et al., 2017; see recent reviews by Djami-Tchatchou et al., 2017; Wang et al., 2017; Islam et al., 2018). To understand the roles of miRNAs within the redox balance-DDR-miRNA triangle, recent literature was consulted to examine the direct or indirect implication of miRNAs in ROS production/scavenging and DDR pathways, based on their predicted/validated target genes.

miRNAs and ROS

As above described, ROS are by-products of cellular metabolic processes that can act as secondary messengers in specific signaling pathways. In humans, miRNAs targeting central regulators of the ROS signaling pathway have been identified, such as the Nuclear Factor Erythroid-Derived 2-Like 2 (Nrf2), or Tumor Necrosis Factor-Alpha (TNFa), and ROS scavengers, such as SOD or CAT (Wang et al., 2014). Similarly, studies in plants have revealed the presence of miRNAs targeting genes involved in ROS production and scavenging (**Table 2**). The influence of miRNAs in these processes can be classified as (1) direct, when directly targeting genes coding for proteins with oxidant or antioxidant properties, and (2) indirect, when the targeted genes affect redox signaling pathways downstream. miR529 is an example of an indirect influence. This miRNA targets some of the genes belonging to the SQUAMOSA promoter-binding protein-like proteins (SPLs), a plant specific transcription factor involved in regulating plant growth and development (Rhoades et al., 2002). Recently developed rice lines overexpressing *MIR529a* have been shown to have increased resistance to oxidative stress imposed by applying exogenous H_2O_2, because of enhanced levels of SOD and peroxidase (POD, POX) enzymes (Yue et al., 2017). The authors demonstrated that the over-accumulation of miR529a resulted in an enhanced seed germination rate, root tip cell viability, chlorophyll retention, and reduced leaf rolling rate during exposure to H_2O_2. Regarding the miR529a targets, out of the five predicted genes (*OsSPL2*, *OsSPL14*, *OsSPL16*, *OsSPL17*, *OsSPL18*) only two, *OsSPL2* and *OsSPL14*, were

TABLE 2 | List of miRNAs targeting genes with roles in ROS production and scavenging.

miRNA	Species	Targeted genes	Related stress	References
miR395	*Arabidopsis thaliana* *Brassica napus* *Oryza sativa* *Nicotiana tabacum*	*ATPS, SULTR2;1*	Nutrient deficiency Heavy metal	Matthewman et al., 2012; Zhang L. W. et al., 2013; Jagadeeswaran et al., 2014; Panda and Sunkar, 2015; Yuan et al., 2016
miR396b	*Poncirus trifoliata*	*ACO*	Cold	Zhang et al., 2016
miR397	*Arabidopsis thaliana* *Oryza sativa* *Lotus japonicus*	*LAC*	Nutrient deficiency H_2O_2	Li et al., 2011; De Luis et al., 2012; Zhang Y. C. et al., 2013; Wang et al., 2014
miR398	*Arabidopsis thaliana* *Vitis vinifera* *Triticum aestivum* *Phaseolus vulgaris* *Medicago truncatula*	*CDS1, CDS2, Nod19, COX5b*	Heavy metal Drought Salinity	Trindade et al., 2010; Naya et al., 2014; Kayıhan et al., 2016; Leng et al., 2017; Li J. et al., 2017; Li L. et al., 2017
miR408	*Arabidopsis thaliana* *Oryza sativa* *Nicotiana tabacum* *Medicago truncatula*	*PCY, PLC, LAC, UCC, UCL8*	Biotic stress Drought Salinity γ-irradiation	Trindade et al., 2010; Zhang et al., 2017; Pan et al., 2018; Song et al., 2018
miR414	*Panicum virgatum*	*CAT isozyme B, PAO, NADH_UbQ/plastoQ_OxRdtase, HSP, COX*	–	Xie et al., 2010
miR474	*Citrus sinensis* *Zea mays*	*PDH, NAD*-dependent malic enzyme	Boron deficiency Submergence	Zhang et al., 2008; Lu et al., 2014
miR477	*Panicum virgatum* *Triticum aestivum*	*Fd-GOGAT*	Drought	Xie et al., 2010; Akdogan et al., 2016
miR528	*Oryza sativa* *Agrostis stolonifera*	*PCY*-like, *LAC, MCOs, GALTs, AO*	Drought Salinity Heavy metals	Liu et al., 2015; Yuan et al., 2015; Wu et al., 2017
miR531	*Panicum virgatum* *Triticum aestivum*	*HSP 17.9, POD52, POX, CYP P450, ACO1*	Environmental pollutants	Xie et al., 2010; Li J. et al., 2017
miR9773	*Triticum aestivum*	*CYP P450*	Environmental pollutants	Li J. et al., 2017
miR1121	*Triticum aestivum*	*CAT-1, POD6, MT3*-like	Environmental pollutants	Li J. et al., 2017
miR9653b	*Triticum aestivum*	*LOX*-like protein	Environmental pollutants	Li J. et al., 2017
miR1132	*Panicum virgatum*	*CYP87A15*	–	Xie et al., 2010
miR1436	*Panicum virgatum* *Oryza sativa*	*POD2*	Heat	Xie et al., 2010; Mangrauthia et al., 2017
miR1535	*Panicum virgatum*	*CYP724B3*	–	Xie et al., 2010
miR2102	*Panicum virgatum* *Oryza sativa*	*CYP P450, SOD, COX VI, POD, ACO1*	Arsenic	Xie et al., 2010; Sharma et al., 2015
PC-5p-213179-14	*Zea mays*	*POD*	Low seed vigor	Gong et al., 2015
PN-2013	*Triticum aestivum*	*MDHAR*	Biotic stress	Feng et al., 2014
novel_miR_120	*Brachypodium distachyon*	*NDH1*α subunit 12	H_2O_2	Lv et al., 2016
novel_miR_4	*Brachypodium distachyon*	*CYP P450 734A1*	H_2O_2	Lv et al., 2016
novel_miR_234	*Brachypodium distachyon*	*FTR*	H_2O_2	Lv et al., 2016
novel_miR_197	*Brachypodium distachyon*	*CYP P450 90D2*	H_2O_2	Lv et al., 2016

downregulated in seedlings overexpressing *MIR529a*, therefore suggesting that these two were the direct targets of miR529a. This also induced the upregulation of other stress-related genes such as *OsCPR5* (Constitutive expression of pathogenesis-related genes 5), proline synthase (Os10g0519700), amino acid kinase (LOC_Os05g38150), peroxidase precursor (LOC_Os04g59150), and *OsVPE3* (Vacuolar processing enzyme-3). Based on these findings, the authors proposed a potential complex network of miR529a-SPLs-downstream genes in the ROS signaling pathway in response to oxidative stress (Yue et al., 2017).

Table 2 summarizes the information related to 23 miRNAs that directly target several genes with roles in ROS production/scavenging in various plant species. Of these, the most studied in relation to oxidative stresses are miR395 and miR398. The predicted and validated targets of miR395 are the ATP sulfurylase (ATPS) and low-affinity sulfate transporters SULTR2;1 (Matthewman et al., 2012; Jagadeeswaran et al., 2014). ATPS catalyzes the activation of sulfate by transferring sulfate to the adenine monophosphate moiety of ATP to form adenosine 5′-phosphosulfate and pyrophosphate (Patron et al., 2008). SULTR2;1 is responsible for the internal transport of sulfate from roots to shoots (Takahashi et al., 2000). The modulation of miR395 thus seems ideal to address the sulfate assimilation pathway and

develop crops with increased efficiency of sulfate uptake (Yuan et al., 2016). A key question is how this is related to redox signaling. When sulfate reaches chloroplasts and mitochondria, it is reduced first to sulphite and then to sulfide, which is essential for the synthesis of cysteine and methionine, two fundamental amino acids for supporting redox reactions in plants. The reduced form of cysteine functions as an electron donor, while its oxidized form acts as an electron acceptor. This different redox state allows to hypothesize a role of redox signaling in inducing nutrient-related or stress-responsive miRNAs. Above all, it refers to the intracellular thiol redox status, which regulates a variety of cellular and molecular events such as the activity of proteins, signal transduction, transcription and several other cellular functions (Panda and Sunkar, 2015). Another well-studied example is miR398, which targets the metal-induced superoxide dismutases, CDS1 and CDS2, in a number of different species (see **Table 2**; Trindade et al., 2010; Naya et al., 2014; Kayıhan et al., 2016; Leng et al., 2017; Li J. et al., 2017; Li L. et al., 2017). Because of its role in regulating this important ROS scavenger enzyme, miR398 has been found to be involved in plant responses to a multitude of stresses, including drought (Trindade et al., 2010), salinity (Feng et al., 2015), metal-induced toxicity (Xu et al., 2013), and other pollutants such as sulfur dioxide (SO_2) (Li L. et al., 2017).

Other miRNAs (e.g., miR414, miR531, miR1121, miR1436, miR2102) that target other ROS scavenging enzymes such as CAT, SOD, POD, and POX have been identified and their involvement in the plant stress response has been proven (Xie et al., 2010; Sharma et al., 2015; Li J. et al., 2017; **Table 2**). A particular example is miR414, which targets a myriad of genes with different functions in plant stress metabolism and antioxidant responses. As shown by Xie et al. (2010) in switchgrass, miR414 was predicted to target 44 different mRNAs, several of which dealing with oxygen/ROS including CAT isozyme B, polyamine oxidase (PAO), cytochrome *c* oxidase (COX), and NADH-ubiquinone oxidoreductase B16.6 subunit (NADH_UbQ/plastoQ_OxRdtase).

miRNAs and DDR

The ability of DDR to sense DNA damage, transduce signals and promote repair, depends on the coordinated action of a series of factors. Of these, the MRN complex represents the first "line of defense" as it acts as a sensor of damage signaling by recruiting DDR-related proteins, including ATM and other mediators, to the DSB sites (Goldstein and Kastan, 2015).

In human cell research, miRNAs are being investigated for their modulator role in the regulation of DDR (for review see Hu and Gatti, 2011; He et al., 2017). For example, miR-18a and miR-412 have been proved to negatively regulate ATM expression and reduce the capacity of DNA damage repair in tumorigenic cells challenged with irradiation or chemotherapy (Song et al., 2011; Mansour et al., 2013). Other studies have demonstrated that miRNAs are involved in the post-transcriptional regulation of p53 (Hu et al., 2010; Kumar et al., 2011), the master-regulator of DDR that drives the fate of the human cell directing it to DNA repair, cell cycle arrest, apoptosis, or senescence. For instance, miR-25 and miR-30d have been shown to interact with p53, and, as a consequence, its downregulation leads to the suppression of some of its target genes (*p21*, *BAX*, *Puma*) resulting in reduced apoptosis (Kumar et al., 2011). Downstream effectors, such as the DNA repair pathways, are also influenced by miRNAs at least in animals, as shown in several studies investigating human cancer cell lines. Moreover, examples of miRNA involvement in NHEJ (e.g., miR-101) or HR repair mechanism (e.g., miR-107, miR-103, miR-222) have been reported in animal and plant cells (Yan et al., 2010; Huang et al., 2013; Neijenhuis et al., 2013). In the case of the hsa-miR-526b, which targets the Ku80 mRNA, in addition to DSB repair, the plant cell cycle progression is also affected in the G_0/G_1 phase (Zhang, 2015).

In plants few studies have addressed the potential role of miRNAs in the regulation of DDR-associated genes. Most of this evidence comes from high-throughput transcriptomic studies dedicated to investigating specific stress responses/adaptations. **Table 3** summarizes a collection of miRNAs predicted to target several genes with different roles in the DDR pathway.

In a study on changes in miRNA expression during magnesium (Mg)-induced starvation in oranges roots, the authors collected different miRNAs affecting several functions, ranging from the antioxidant response, adaptation to low-phosphorus and activation of transport-related genes, to DNA repair (Liang et al., 2017). The study identified the MUTL-homolog 1 (MLH1) and MRE11 as targets of miR5176 and miR5261, respectively. The *MLH1* gene is part of the MMR pathway, one of the DNA repair defense systems responsible for maintaining genome integrity during cell division. Previous studies in yeast have identified four MutL homologs that form functionally distinct heterodimers, of which Mlh1/Pms1 and Mlh1/Mlh2 are involved in the correction of different types of DNA mismatches (Wang et al., 1999). In plants, the MLH1 has been less investigated compared with other MutL/MutS homologs. However, interaction between MLH1 and MLH3 has been shown to be required for the formation of double Holliday junctions and normal levels of meiotic crossovers in Arabidopsis plants (Jackson et al., 2006). Thus, identifying a miRNA capable of suppressing the activity of MLH1 would also help to better clarify the functions of this gene. The particular case of miR5176 showed that its induction under Mg-deprived conditions resulted in the activation rather than the inhibition of MLH1 associated with enhanced MMR activity in response to Mg-deficiency (Liang et al., 2017). This could be due to other post-transcriptional modifications or the activation of alternative regulatory mechanisms. In addition, the *MRE11* gene that encodes DNA repair and meiosis proteins belonging to the MNR complex, was identified as being targeted by miR5261, and induced in Mg-deprived roots. In this case, the down-regulation of miR5261 resulted in enhanced levels of MRE11

TABLE 3 | List of miRNAs targeting genes with roles in DNA damage response.

miRNA	Species	Targeted genes	Related stress	References
miR1127a	*Triticum aestivum*	SMARCA3L3	–	Sun et al., 2018
miR2275	*Triticum aestivum* *Prunus persica*	CAF1	Drought	Esmaeili et al., 2017; Sun et al., 2018
miR122c-3p	*Triticum aestivum*	XPB2	–	Sun et al., 2018
miR5179	*Citrus sinensis*	MUTL-homolog 1	Mg-deficiency	Liang et al., 2017
miR5261	*Citrus sinensis*	MRE11	Mg-deficiency	Liang et al., 2017
miR528b	*Hordeum bulbosum*	RFA1C	Salinity	Liu and Sun, 2017
miR403	*Helianthus annuus*	AGO1, AGO2	Salinity	Ebrahimi Khaksefidi et al., 2015; Kumar et al., 2018
miR2102	*Panicum virgatum* *Oryza sativa*	TFIID subunit 10	Arsenic	Xie et al., 2010; Sharma et al., 2015
miR477	*Panicum virgatum* *Triticum aestivum*	RAD23	Drought	Xie et al., 2010; Akdogan et al., 2016
novel-mir_222	*Brachypodium distachyon*	TFIID subunit 12	H_2O_2	Lv et al., 2016
novel-mir_120	*Brachypodium distachyon*	TFIID subunit 12	H_2O_2	Lv et al., 2016
novel-mir_98	*Brachypodium distachyon*	TFIID subunit 12	H_2O_2	Lv et al., 2016
novel-mir_69	*Brachypodium distachyon*	RAD50	H_2O_2	Lv et al., 2016
novel-mir_147	*Brachypodium distachyon*	SMUBP-2	H_2O_2	Lv et al., 2016
novel-mir_4	*Brachypodium distachyon*	SAGA29	H_2O_2	Lv et al., 2016
miR414	*Oryza sativa*	OsABP helicase	Salinity γ-irradiation	Macovei and Tuteja, 2012; Macovei and Tuteja, 2013
miR408	*Oryza sativa*	OsDSHCT helicase	Salinity γ-irradiation	Macovei and Tuteja, 2012; Macovei and Tuteja, 2013
miR164e	*Oryza sativa*	OsDBH helicase	Salinity γ-irradiation	Macovei and Tuteja, 2012; Macovei and Tuteja, 2013

and, as a consequence, better detection of DNA damage and repair of DSBs.

In another study aimed at determining miRNAs responsive to H_2O_2 during seedling development in *Brachypodium distachyon*, a novel miRNA called novel_mir_69 was identified as targeting the *RAD50* mRNA (Lv et al., 2016). Using next generation high-throughput sequencing, a total of 144 known and 221 new miRNAs were identified as being responsive to H_2O_2-induced stress in *B. distachyon*. In addition to *RAD50*, other genes with a role in DNA damage repair were shown to be targeted by several other newly identified miRNAs in this study. For instance, the DNA-binding protein encoded by *SMUBP-2* was predicted to be targeted by novel_mir_147, the novel_mir_4 targeting the SAGA-associated factor 29 homolog, while the transcription initiation factor IID (TFIID) was predicted to be targeted by novel_mir_120. The SMUBP-2 is a transcription regulator which also has a 5′ to 3′ helicase activity. Its RH3 helicase domain and AN1-like zinc finger domain have been shown to bind single-stranded DNA (Lim et al., 2012). The SAGA-associated factor 29 homolog is a chromatin reader component of the transcription regulatory histone acetylation (HAT) complex (Kaldis et al., 2011). On the other hand, TFIID is a key component of the transcription pre-initiation complex (PIC), responsible for recognizing and binding to specific promoter DNA sequences (e.g., TATA elements). Studies on yeast have demonstrated that both TFIID and SAGA can be sequentially recruited at the DNA damage site in a differential manner, based on the type of stress induced (Ghosh and Pugh, 2011). For instance, when the methylmethane sulphonate mutagenic agent was used, the induced genes underwent transcription complex assembly sequentially, first through SAGA and then through a slower TFIID recruitment. However, when heat shock was applied, the induced genes used both the SAGA and TFIID pathways rapidly and in parallel. Similarly, studies in plants have demonstrated that TFIID associates with essential proteins involved in DNA repair and chromatin remodeling, such as MRE11 and TAF1 (TATA-binding protein Associated Factor 1, histone acetyltransferase), in an attempt to maintain genome integrity under genotoxic stress conditions (Waterworth et al., 2015).

The fact that miRNAs were predicted to directly or indirectly interact with chromatin remodeling associated genes further adds to the complicated layers of regulation of this complex phenomenon. In a bioinformatics study on switchgrass, TFIID mRNA was predicted to be targeted by miRNAs (miR2102) (Xie et al., 2010). In the same study, another DNA repair gene, namely *RAD23*, was predicted to be targeted by miR477. The *RAD23* gene, encoding for the UV excision repair protein RAD23 homolog A, is involved in the NER pathway. By interacting with several other components of the DNA repair machinery, it also plays an important role in BER DNA damage recognition (Sturm and Lienhard, 1998). In Arabidopsis, RAD23 have also been demonstrated to have an essential role in the cell cycle, morphology, and fertility of plants through their involvement in ubiquitination pathways (Farmer et al., 2010). Another component of the NER pathway

predicted to be targeted by miRNAs is *XPB2* (homolog of *Xeroderma pigmentosum* complementation group B2). In a transcriptome analysis performed during anther development in male sterile wheat lines, *XPB2*, a DNA repair helicase, was shown to be targeted by tae-miR1122c-3p (Sun et al., 2018). The induced expression of *XPB2*, acting as a DNA damage detector, has been suggested to be necessary for DNA damage repair during pollen formation. It is worth noting that this study used a particular wheat line (337S), which is sensitive to both long-day-length/high-temperature and short-day-length/low-temperature, to investigate the miRNA involvement in the regulation of male sterility by looking at the pre-meiotic and meiotic cell formation (Sun et al., 2018). Besides *XPB2*, other DNA repair and chromatin remodeling associated genes have been identified as targets of miRNAs. For instance, tae-miR2275 targeted the *CAF1* (CCR4-associated factor 1), involved in early meiosis, whereas tae-miR1127a targeted the *SMARCA3L3* (a new member of SWI/SNF factor SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A, member 3-like 3), believed to be involved in the progression of meiosis in male reproductive cells. In yeast, the CCR4-Not (Carbon Catabolite Repressed 4-Negative on TATA-less) complex has been shown to be involved in replication stress and DNA damage repair, as well as maintaining heterochromatin integrity (Mulder et al., 2005; Cotobal et al., 2015). The SWI/SNF chromatin-remodeling complex is, instead, an essential component of chromatin remodeling, and its involvement in DNA damage response is dependent on the CCR4–Not complex (Mulder et al., 2005). By showing interactions between tae-miR2275-*CAF1* and tae-miR1127a-*SMARCA3L3,* this study demonstrated that the diversified roles of SMARCA3L3 and CAF1 in DNA repair and chromatin remodeling helped to maintain chromatin and genome integrity during meiosis (Sun et al., 2018).

Other miRNAs that putatively control different helicase genes have been identified by *in silico* analysis in rice (Umate and Tuteja, 2010). Of these, osa-MIR414, osa-MIR408 and osa-MIR164e have been experimentally validated as targeting the *OsABP* (ATP-Binding Protein), *OsDSHCT* (DOB1/SK12/helY-like DEAD-box Helicase), and *OsDBH* (DEAD-Box Helicase) genes (Macovei and Tuteja, 2012). The expression of miRNAs and their targeted genes correlated negatively in response to salinity stress and gamma-irradiation treatments, which caused DNA, damage (Macovei and Tuteja, 2012, 2013). Given that helicases are enzymes that catalyze the separation of double-stranded nucleic acids in an energy-dependent manner, they are involved in a wide range of processes such as recombination, replication and translation initiation, double-strand break repair, maintenance of telomere length, nucleotide excision repair, and cell division and proliferation (Tuteja, 2003). Hence, by targeting a wide range of helicases, as shown by the literature cited here, miRNAs are responsible for regulating all this array of processes associated with helicase activities.

An interesting aspect of miRNAs is their capacity to regulate their own biogenesis. This happens by targeting ARGONAUTE genes (AGO1 and AGO2), as in the case of miR403 and miR172 (Ebrahimi Khaksefidi et al., 2015). Aside from their involvement in small RNA pathways and epigenetic silencing phenomena (Schraivogel and Meister, 2014), AGOs have also been shown to be associated directly or indirectly with DNA repair (Oliver et al., 2014). The particular case of miR403 and miR172 shows that in addition to targeting AGO, they also interact with DML1 and DML3 (involved in DNA methylation), thus suggesting the multiple role of these miRNAs in small RNA pathways and DNA methylation (Ebrahimi Khaksefidi et al., 2015).

CONCLUSION

This review has explored the interconnections between the molecular mechanisms controlling the cell redox balance and gene expression regulation, occurring at transcriptional and post-transcriptional levels, as well as the maintenance of genome integrity (**Figure 1**). In particular, evidence here reported, underline the influence of the redox signaling in the modulation of molecular pathways activated in response to developmental and environmental stimuli. Interestingly, specific players involved in redox sensing and homeostasis, influence plant metabolism at different levels. During evolution, plants, as all other living organisms, have developed capability for using specific molecular players in a cross-cutting manner both in developmental processes, in defense responses activated by environmental stimuli and in DNA replication and repair. GSH and correlated thiol systems represent a case in point of key actors controlling the redox buffering capability of plant nuclei and they are crucial also for DNA replication and repair, and consequently cell cycle progression, as well as for the regulation of gene expression in different contexts (Diaz-Vivancos et al., 2010; Martins et al., 2018; Ratajczak et al., 2019). Moreover, numerous TFs, regulating the expression of genes involved in plant development, DDR or in the activation of stress-related responses, are described to be redox-regulated. The activity of these TFs is mainly influence d by alterations in the cell redox balance, which lead to conformational changes and their possible subcellular re-location. Recently, evidence of the involvement of a continuously increasing number of miRNAs in several processes is opening new scenarios on the complexity of redox signaling and homeostasis. Although some miRNAs targeting genes with different roles related to defense systems, development and DDR pathways have been predicted or validated in different plant species, this field requires further investigation. Interestingly, some miRNAs have been predicted to target genes belonging to the above-indicated pathways. Examples include miR408 and miR414, which target the helicases involved in DNA repair as well as several genes implicated in the redox system (see **Tables 2**, **3**). Similarly, miR528 is predicted to target RFA1C (replication A 70 KDa DNA-binding subunit C), involved in DNA replication and efficient DNA repair and recombination (Longhese et al., 1994), as well as antioxidant-related genes (e.g., phytochromes, oxidases). Switchgrass miR477 has also been shown to target the Rad23 DNA repair associated factor as well as Ferredoxin-Dependent Glutamine-Oxoglutarate Amidotransferase (Fd-GOGAT), acting as electron donor in

glutamate metabolism (Xie et al., 2010). The evidence here reported highlight an interconnectivity between the redox and DDR pathways created by a network of miRNAs. Further studies aimed at clarifying these complex regulatory networks are strongly encouraged.

AUTHOR CONTRIBUTIONS

AB, AM, and VL drafted the manuscript. SC, CG, AM, and VL wrote the manuscript. SC and AM created the figures and tables. AB and LD revised and critically improved the manuscript.

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Argonaute Proteins: Why Are They So Important for the Legume–Rhizobia Symbiosis?

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Unlike most other land plants, legumes can fulfill their nitrogen needs through the establishment of symbioses with nitrogen-fixing soil bacteria (rhizobia). Through this symbiosis, fixed nitrogen is incorporated into the food chain. Because of this ecological relevance, the genetic mechanisms underlying the establishment of the legume–rhizobia symbiosis (LRS) have been extensively studied over the past decades. During this time, different types of regulators of this symbiosis have been discovered and characterized. A growing number of studies have demonstrated the participation of different types of small RNAs, including microRNAs, in the different stages of this symbiosis. The involvement of small RNAs also indicates that Argonaute (AGO) proteins participate in the regulation of the LRS. However, despite this obvious role, the relevance of AGO proteins in the LRS has been overlooked and understudied. Here, we discuss and hypothesize the likely participation of AGO proteins in the regulation of the different steps that enable the establishment of the LRS. We also briefly review and discuss whether rhizobial symbiosis induces DNA damages in the legume host. Understanding the different levels of LRS regulation could lead to the development of improved nitrogen fixation efficiency to enhance sustainable agriculture, thereby reducing dependence on inorganic fertilizers.

Keywords: argonaute proteins, legumes, symbiosis, microRNAs, small RNAs

INTRODUCTION

The symbiosis between legumes and rhizobia is of considerable ecological importance because through it, fixed nitrogen (e.g., ammonium) is incorporated into the food chain (Castro-Guerrero et al., 2016). In this context, it has been estimated that the legume–rhizobia symbiosis fixes 60 million metric tons of nitrogen worldwide (Smil, 1999). As symbiotic nitrogen fixation also plays essential roles in soil function, nutrient and water cycling, and food security, its exploitation and improvement in crop plants can promote lower input sustainable agriculture (Ferguson et al., 2019a).

To establish this symbiosis, a molecular dialogue between legumes and rhizobia is required (Venkateshwaran et al., 2013). This dialogue implies the interchange of diffusible signals, which includes legume-derived flavonoids and rhizobia-secreted lipochito-oligosaccharides (LCOs) with specific chemical decorations, named Nodulation Factors (NFs) (Dénarié et al., 1996). Upon NFs

perception by the legume host, a series of molecular events is activated, enabling rhizobial infection and nodule formation (Venkateshwaran et al., 2013).

Legume–rhizobia symbiosis (LRS) is regulated at the transcriptional, posttranscriptional, and posttranslational level (Venkateshwaran et al., 2013). For instance, it has been demonstrated that the transcription factor (TF) Nodule Inception (NIN) controls rhizobial root infection, colonization, and nodule formation (Liu CW et al., 2019; Liu J et al., 2019). NIN also activates the expression of the *CLE ROOT SIGNALING1* (*CLE-RS1*) and *CLE-RS2* peptides in *Lotus japonicus* (Soyano et al., 2014). These two CLE peptides participate in the Autoregulation of Nodulation (AON) process, which limits the number of nodules (Ferguson et al., 2019b).

MicroRNAs (miRNAs), which are small regulatory RNA molecules, play a substantial role in the posttranscriptional regulation of LRS (Moran et al., 2017). For example, it has been demonstrated that miRNAs miR166 and miR169 regulate nodule development (Combier et al., 2006; Boualem et al., 2008) in *Medicago truncatula*. However, miRNAs not only regulate nodule development, but they also participate earlier in the rhizobial infection process (Bazin et al., 2012). The involvement of miRNAs, and likely other small RNAs (sRNAs), in the LRS strongly implicates the participation of Argonaute (AGO) proteins, which together form so-called RNA-induced silencing complexes (RISCs). We recently reported that AGO5 participates in the rhizobial infection process in both *Phaseolus vulgaris* (common bean) and *Glycine max* (soybean) (Reyero-Saavedra et al., 2017). Despite this evidence, the involvement of AGO proteins in LRS has been largely overlooked.

Here, we briefly recapitulate the genetic control of LRS by TFs and miRNAs. Likewise, based on the role of different small RNAs (sRNAs) and some AGO proteins in the regulation of both plant development and plant–pathogen interactions, we hypothesize the stages of this symbiosis where AGO proteins might play a role. Finally, we also discuss whether rhizobial symbiosis causes DNA damage in the legume host. By improving our understanding of the different levels of LRS regulation, we may be able to enhance symbiotic nitrogen fixation efficiency in crop legumes.

GENETIC REGULATION OF LEGUME–RHIZOBIA SYMBIOSIS

NFs are detected by two LysM-type receptor kinases, named NFs Perception (NFP) and LysM-domain Receptor-Like Kinase3 (LYK3), in *M. truncatula* and NFs Receptor5 (NFR5) and NFR1 in *L. japonicus* (Limpens et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). Both NFP/NFR5 and LYK3/NFR1 receptors have a similar structure, which includes an extracellular domain composed of three LysM domains, a transmembrane domain, and an intracellular kinase domain. These two receptors are essential for legume–rhizobial communication, and they may have evolved independently from two different ancestral receptors, which were likely involved in the perception of Mycorrization (Myc)-LCOs (De Mita et al., 2014). Myc-LCOs are signal molecules released by endomycorrhizal fungi and are required for most land plants to engage in symbiosis with these beneficial microbes (Maillet et al., 2011). Interestingly, Myc-LCOs and NFs are structurally very similar, which reinforces the hypothesis that NF receptors evolved from ancestral receptors involved in the perception of Myc-LCOs. The evolution of the NF's extracellular domain arguably provided high specificity to the rhizobial symbiosis; it has been demonstrated that the evolution of the second LysM domain contributed to ligand binding, whereas the first LysM domain contributed to ligand specificity (De Mita et al., 2014).

Upon perception of NFs *via* the receptors NFP/NFR5 and LYK3/NFR1, a series of molecular events, including protein phosphorylation, are triggered (Broghammer et al., 2012). The phosphorylation of proteins is crucial to decipher the NFs signal. For example, one of the phosphorylated proteins playing a role in this symbiosis is 3-hydroxy-3-methylglutaryl coenzyme A reductase1 (HMGR1) (Kevei et al., 2007). HMGR1 participates in mevalonate biosynthesis, and it has been demonstrated that mevalonate is sufficient to trigger calcium oscillations in the nuclear region, also known as calcium spiking (Venkateshwaran et al., 2015). Calcium spiking is a crucial signature to establish rhizobial symbiosis. Membrane ion channel mutants, such as *L. japonicus castor* and *pollux* and the *M. truncatula* mutant that *does not make infections1* (*dmi1*), are unable to activate calcium spiking and therefore fail to nodulate (Imaizumi-Anraku et al., 2005).

Calcium spiking is further decoded by a calcium/calmodulin (Ca^{+2}/CaM)-dependent protein kinase (CCaMK/DMI3) (Lévy et al., 2004). Upon activation, CCaMK/DMI3 immediately phosphorylates the transcriptional activator Interacting Protein of DMI3 (IPD3)/CYCLOPS (Singh et al., 2014). In turn, IPD3/CYCLOPS activates the expression of *NIN*, which subsequently promotes the expression of the *Nuclear Factor Y* (*NF-Y*) complexes *NF-YA* and *NF-YB* (Soyano et al., 2013). The coordinated action of these TFs and the interplay of the TF Nodulation Signaling Pathway2 (NSP2)/NSP1, Ethylene Response Factor Required for Nodulation1 (ERN1), and ERN2 lead to the transcriptional activation of symbiosis-related genes participating in the rhizobial infection process (Genre and Russo, 2016). Some of the genes activated by this transcriptional node are *Early Nodulin11* (*ENOD11*), which is involved in the infection processes (Journet et al., 2001), and the *Flotillins* (*FLOT*) *FLOT2* and *FLOT4*, which are involved in the formation of the infection thread, a tubular structure essential for rhizobial infection of the root cells (Haney and Long, 2010) (**Figure 1**).

In parallel with the activation of the molecular events leading to the infection/colonization of the root by the rhizobia, legumes activate a second genetic program that is required for nodule development (Oldroyd et al., 2011; Plet et al., 2011). It has been demonstrated that a delicate balance between the phytohormones auxin and cytokinin activates this genetic program (Hirch et al., 1989; Van Zeijl et al., 2015; Gamas et al., 2017). The activation of this genetic program begins with the inhibition of polar auxin transport, which leads to the accumulation of cytokinins in root cortical cells (Nadzieja et al., 2018). Cytokinins are then detected in root cortical cells through the receptor Cytokinin Response1 (CRE1)/Lotus Histidine Kinase1 (LHK1) (Plet et al., 2011). Interestingly, upon cytokinin perception, NIN and NSP2/NSP1 are also activated, controlling the expression of genes involved in the development of the nodule (Madsen et al., 2010).

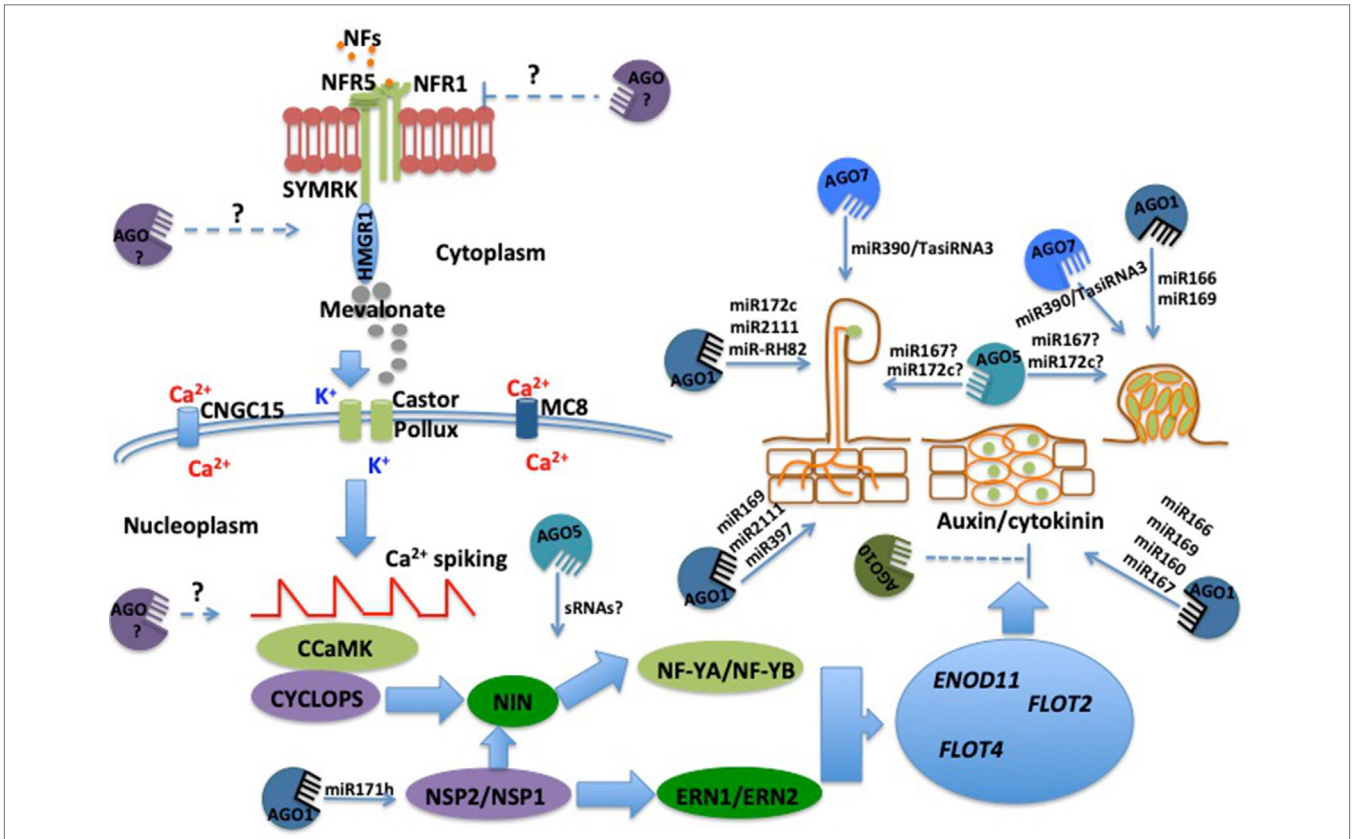

FIGURE 1 | Participation of Argonaute (AGO) proteins in different stages of the legume–rhizobia symbiosis (LRS) According to several reports, different AGO proteins may participate in each stage of the LRS. Although several miRNAs have been identified a few hours upon NF perception, there is no experimental evidence indicating that they regulate very early stages of LRS, such as NFs perception and activation of calcium spiking. However, there is solid evidence supporting the participation of both sRNAs and different AGO proteins in rhizobial infection and the development of both nodule meristems and root nodules. Dashed lines indicate the potential participation of AGO proteins and sRNAs in the LRS.

Although this symbiosis provides fixed nitrogen to the plant, this process demands a significant amount of energy from legumes. Because of this carbon demand, legumes tightly regulate the number of nodules via AON. In *L. japonicus,* AON is systemically regulated by the CLE-RS1 and CLE-RS2 peptides (Soyano et al., 2013; Ferguson et al., 2019b). These two CLE peptides travel from the root to the shoots where they are detected by the receptor Hypernodulation Aberrant Root formation1 (HAR1) (Nishimura et al., 2002). Upon perception of CLE peptides, a signal molecule, likely a shoot-derived cytokinin or the miRNA miR2111, is produced and sent to the roots (Tsikou et al., 2018; Ferguson et al., 2019b). The perception of this shoot-derived molecule in the roots then triggers the inhibition of nodule development.

ROLE OF miRNAS IN THE ESTABLISHMENT OF THE LEGUME–RHIZOBIA SYMBIOSIS

The first miRNAs known to be involved in the LRS were miR169 and miR166, which regulate meristem maintenance, bacterial release, and vascular differentiation in both roots and nodules of *M. truncatula* plants (Combier et al., 2006; Boualem et al., 2008). MiR169 and miR166 regulate these stages of LRS through the modulation of the expression of the TF genes *NF-YA1* (formerly called *HAP2-1* for *HAPLESS2-1*) and *class-III homeodomain-leucin zipper* (*HD-ZIPIII*), respectively (Combier et al., 2006; Boualem et al., 2008; Laloum et al., 2013). Since the publication of these two studies, a large number of symbiosis-responsive miRNAs has been identified in different stages of LRS. For instance, Subramanian et al. (2008) reported many miRNAs that were differentially regulated after 3 h of rhizobial inoculation in soybean (**Table 1**).

Because LRS is initiated in root hairs, Formey et al. (2016) hypothesized that root hair miRNA expression analysis after 6 h of NFs treatment could identify regulators of early events of rhizobial infection. As a result, Formey et al. (2016) identified six symbiosis-responsive miRNAs in the common bean. Interestingly, one of the identified miRNAs was the root hair-specific miR-RH82. This observation suggests that this novel miRNA might play an essential role in the early stages of the LRS (Formey et al., 2016).

Although several studies report differential expression of miRNAs during the first hours of legume–rhizobia interaction, there is limited experimental evidence to indicate that they regulate very early symbiotic events, such as calcium spiking.

TABLE 1 | Differentially regulated plant miRNAs and their corresponding target genes during the early stages of the legume–rhizobia symbiosis.

Reference	hpi	Regulation	miRNA	Target Gene Name
Subramanian et al., 2008	3	Up	miR168	*Argonaute 1*
			miR172	*Apetala 2 like*
			miR159	*Auxin Responsive Factor like*
			miR393	*Transport Inhibitor Response 1*
		Down	miR160	*Auxin Responsive Factor 10,16,17*
			miR164	*NAC domain containing protein 1*
			miR166	*Class III homeodomain leucine zipper*
			miR169	*Nuclear Factor YA-1* (*Hapless 2-1)*
			miR396	*Growth-Regulating Factors*
Formey et al., 2016	6	Up	miR171a	*Nodulation-signaling pathway 2*
			miR398b-3p	*Cu/Zn Superoxide Dismutase 1/Nodulin 19*
		Down	miR171a-3p	*GRAS family transcription factor*
			miR398c	ND
			miR482b-3p	*Nucleotide-Binding Site–Leucine-Rich Repeat*
			miR-RH82	ND

hpi, hours postinoculation; ND, nondetermined target gene.

However, miRNAs have been identified that participate in rhizobial preinfection and infection processes, including miR171c and miR397 in *L. japonicus* (**Figure 1**) (De Luis et al., 2012). Interestingly, miR171c has been shown to target transcripts of the TF gene *NSP2*, which is crucial for the preinfection and infection process (De Luis et al., 2012). To provide evidence supporting the role of these two miRNAs in the rhizobial infection process, De Luis et al. (2012) made use of *L. japonicus snf1* and *snf2* mutants, which produce autoactive versions of the CCaMK and the cytokinin receptor LHK1, respectively. These two mutants can develop nodules in the absence of rhizobia (spontaneous nodules), but they also form infected functional nodules upon rhizobial inoculation (Tirichine et al., 2006a; Tirichine et al., 2006b; Tirichine et al., 2007). By using these mutants, De Luis et al. (2012) demonstrated that miR171c and miR397 significantly accumulate in infected nodules of *snf* mutants but not in spontaneous nodules, suggesting that these miRNAs might play a role in the rhizobial infection process. Another early-acting miRNA is miR172c, which has been demonstrated to target transcripts of the TF gene *APETALA2-1* (*AP2-1*) and plays a role in rhizobia-induced root hair deformation in the common bean (Nova-Franco et al., 2015). In addition, miR172c has also been characterized in soybean, where it acts as a regulator of early nodulins during nodule initiation through the TF Nodule Number Control1 (GmNNC1) (Wang et al., 2014). In the context of the systemic AON mechanism activated upon rhizobial infection, one candidate for the induced shoot-derived inhibitor (SDI) of nodulation could be miR2111, which targets transcripts of the *F-box* gene *Too Much Love*, a crucial regulator of rhizobial infection and nodule number in *L. japonicus* (Tsikou et al., 2018; Ferguson et al., 2019b).

Moving beyond the early stages of infection, several miRNAs participating in nodule development have been reported. To initiate the formation of the nodule meristem and nodule, a delicate balance between auxin and cytokinin is required (Oldroyd et al., 2011; Plet et al., 2011). In soybean plants, miR160 is essential to modulate the levels of these two phytohormones for nodule development (Turner et al., 2013; Nizampatnam et al., 2015). Recently, it has also been demonstrated that the miR390/Trans-Actin Short Interference RNA3 module negatively regulates both rhizobial infection and nodule organogenesis in *M. truncatula* (Hobecker et al., 2017).

ARGONAUTE PROTEINS IN SYMBIOSIS

AGO proteins are present in eukaryotes, and they participate in many biological processes, including interactions with the environment. AGO proteins are characterized by the presence of four domains: a variable N-terminal domain and conserved PAZ (PIWI-ARGONAUTE-ZWILLE), MID (middle), and PIWI domains (Tolia and Joshua-Tor, 2007). The PAZ domain binds sRNAs, whereas the MID domain specifically recognizes the 5' nucleotide of sRNAs. The PIWI domain adopts an RNase H-like fold, enabling most AGO proteins to cleave target messenger RNAs complementary to the bound sRNAs (Song et al., 2004). The number of AGO proteins present in plants is variable and is plant species-dependent (**Figure 2**). For instance, the *Arabidopsis thaliana* genome encodes 10 AGO proteins, whereas the soybean and the common bean genomes encode 22 and 14 AGO proteins, respectively (Liu et al., 2014; Reyero-Saavedra et al., 2017). Despite this diversity of AGO proteins in flowering plants, these proteins can be grouped into three major phylogenetic clades: AGO1/5/10, AGO2/3/7, and AGO4/6/8/9 (**Figure 2**), with AGO1 being the founding member of the *AGO* gene family (Zhang et al., 2015).

Recent evidence indicates that AGO proteins respond to environmental stimuli (Manavella et al., 2019). The direct involvement of plant AGO proteins in biotic interactions is also well known, mainly for plant defense against bacteria and virus

(Raja et al., 2008; Carbonell and Carrington, 2015; Fátyol et al., 2016). However, the role of AGO proteins in the regulation of mutualistic interactions, such as symbiosis, in animals as well as plants is poorly documented. In plants, there is only one report on this topic, which demonstrates the importance of AGO5 in LRS regulation (Reyero-Saavedra et al., 2017). Despite these knowledge gaps, several studies provide evidence that converges on the importance of AGO proteins in LRS. In this section, we aim to compile exhaustive information about legume AGO protein clades and hypothesize roles for some of them in each stage of LRS.

Ago1/5/10 Clade

As many miRNAs have been reported as regulators of different stages of LRS (**Table 2**), AGO1 is clearly involved in this symbiotic process. For example, very recently, it was demonstrated that soybean AGO1 is hijacked by three rhizobial tRNA-derived small RNA fragments to regulate the expression of three plant genes involved in both rhizobial infection and nodule development (Ren et al., 2019). Other members of the AGO1/5/10 clade may also be involved in LRS regulation. The clearest evidence of AGO regulation of nodulation involves *AGO5* (Reyero-Saavedra et al., 2017), which is upregulated 3 h after rhizobial inoculation in common bean and soybean roots. Furthermore, AGO5 is required for rhizobia-induced root hair deformation and nodule development (Reyero-Saavedra et al., 2017). One possible explanation for this comes from *A. thaliana*, in which AGO5 associates with miR167 and miR172c (Mi et al., 2008). In legumes, miR167 and miR172c have been shown to participate in nodule development through the regulation of the *AUXIN RESPONSE FACTOR8* and *AP2-1* genes, respectively (Nova-Franco et al., 2015; Wang et al., 2015). Beyond the well-studied AGO1 protein, AGO5 is

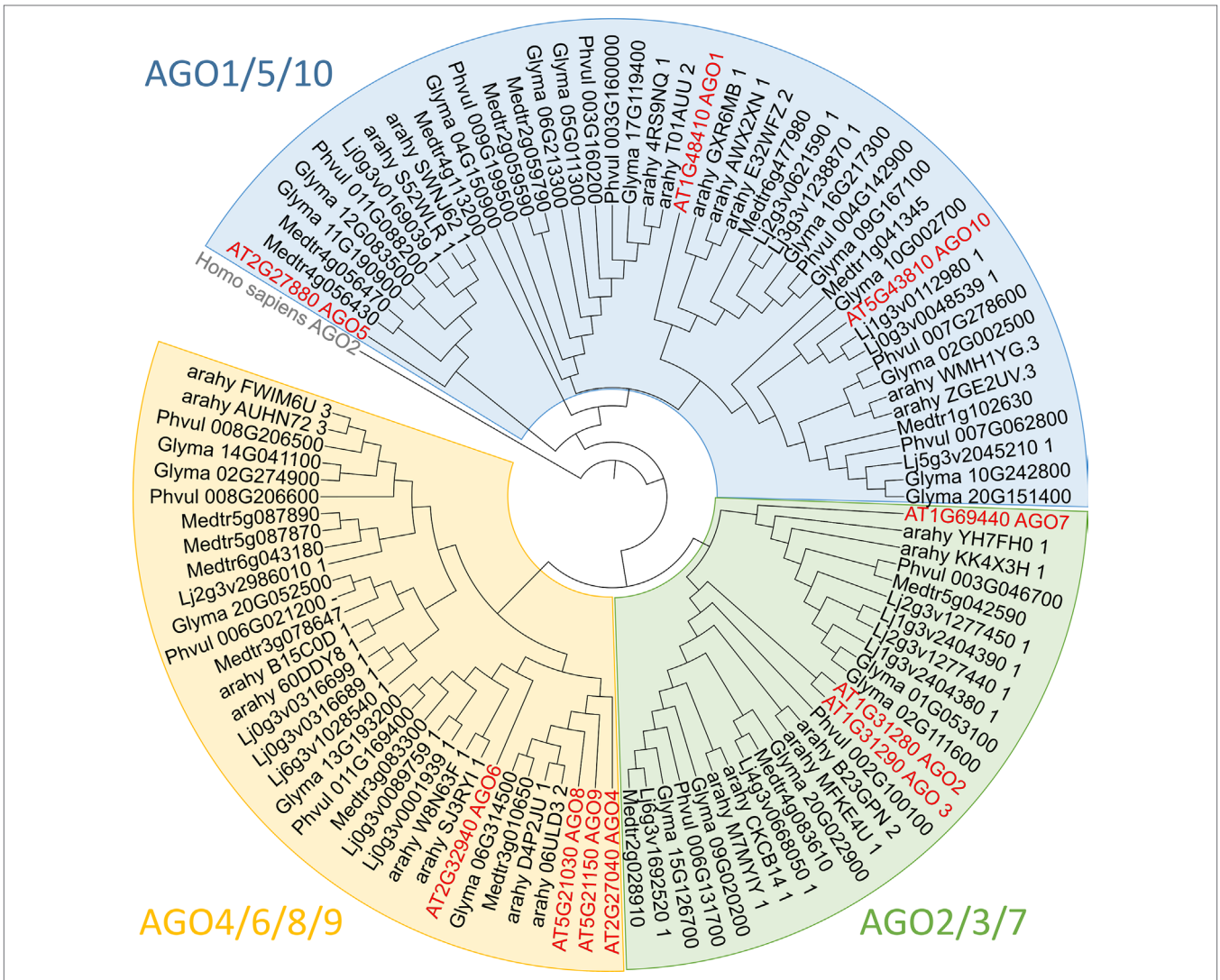

FIGURE 2 | Phylogenetic analysis of legume Argonaute (AGO) family proteins The protein sequences of selected AGOs were obtained from JGI Phytozome v. 12.1.6 (https://phytozome.jgi.doe.gov), Lotus Base (https://lotus.au.dk), and PeanutBase (https://peanutbase.org) and aligned using MAFFT online service v7.427 (Katoh et al., 2017) with FFT-NS-i option set. The phylogenetic tree was constructed using the average linkage (UPGMA) method and designed thanks to iTOL 4.4.2. Abbreviations for selected species are as follows: Medtr, *Medicago truncatula*; Lj, *Lotus japonicus*; Glyma, *Glycine max*; Phvul, *Phaseolus vulgaris*; arahy, *Arachis hypgaea*; AT, *Arabidopsis thaliana.*

the first member of the AGO family that has been demonstrated as a regulator of LRS (Reyero-Saavedra et al., 2017).

AGO10 may also be implicated in the regulation of LRS. It has been reported that AGO10 is capable of sequestering small RNAs, which consequently are not able to associate with their usual corresponding AGO family member (Zhu et al., 2011). This mechanism is involved in regulating the shoot apical meristem (SAM) in *Arabidopsis* (Zhou et al., 2015). To promote SAM differentiation, the action of miR166/165 on their target, which encodes the HD-ZIP III transcription factor, must be suppressed. To achieve this control, plants have selected a regulation system based on the sequestration of miR165/166 by AGO10, which has a higher affinity for these miRNAs than AGO1 and can promote their degradation (Yu et al., 2017). Although this mechanism has not been demonstrated directly in root apical meristem differentiation, some evidence suggests that it could be involved (Ma et al., 2017). In addition, the AGO10 regulatory mechanism is considered an ancient and ubiquitous process in land plant organ development. In *M. truncatula*, the miR166/*HD-ZIP III* node regulates both lateral root and nodule formation through the control of the apical region (Boualem et al., 2008). If the regulation of miR166/*HD-ZIP III* node by AGO10 proteins is a general mechanism, it is tempting to speculate that nodule development could also be controlled in this way. In support of this possible role in LRS, transcripts of *AGO10* group member genes in *M. truncatula*, *Glycine max,* and *P. vulgaris* are upregulated in nodules compared to root tissues (Phytozome v. 12.1.6). This reinforces the hypothesis that AGO10 could be a player in the regulation of nodule development.

AGO2/3/7 Clade

Beyond the phylogenetic grouping, members of the *AGO2/3/7* clade seem to be connected by an involvement in plant defense, employing different regulation mechanisms (Zhang et al., 2011; Fang and Qi, 2016; Rodríguez-Leal et al., 2016). Because AGO2 and AGO3 members are difficult to distinguish in legumes, due to the phylogenetically clustering of the two members by species of origin and not by member type (Zhang et al., 2015), here we focus on the "AGO2/3" group and AGO7 (**Figure 2**).

In *A. thaliana*, AGO2 is a key player in both antiviral defense and antibacterial immune response (Zhang et al., 2011; Carbonell and Carrington, 2015). Moreover, AGO2 is the only member of the *A. thaliana* AGO family reported as highly induced during *Pseudomonas syringae* infection (Zhang et al., 2011). AGO2 acts in this process by loading miR393b*, which targets transcripts of the gene *MEMB12* encoding a Golgi-localized, SDS-resistant, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), and then modulates the exocytosis of antimicrobial Pathogenesis-Related (PR) proteins. LRS is intimately linked to plant immunity (Toth and Stacey, 2015), and PR proteins seem to regulate the rhizobial infection process in soybean and *L. japonicus* (Bartsev et al., 2004; Hayashi et al., 2014). In this context, the involvement of AGO2/3 in the regulation of LRS should be considered. Supporting this hypothesis, AGO2/3 homologs in *M. truncatula*, *G. max,* and *P. vulgaris* are upregulated in nodules compared to root tissues (Phytozome v. 12.1.6). In addition, analysis of legumes AGO proteins shows that AGO2 has undergone gene duplication in *M. truncatula*, *G. max*, and *L. japonicus* (Bustos-Sanmamed et al., 2013) (**Figure 2**). This gene duplication of AGO2/3 suggests that the AGO2/3 isoforms may have diverged in their biological function and could be involved in novel processes, including LRS regulation.

AGO3 is one of the least studied members of the AGO family in plants and, to date, poor information is available about its functionality. Minoia and collaborators (2014) revealed that AGO3 binds siRNAs derived from potato spindle tuber viroid and could be involved in the defense against this pathogen. Similarly, in a recent preprint, Jullien and collaborators (2018) suggest a role of AGO3 in antiviral defense based on its confinement to vascular structures and the fact that most plant viruses use the phloem for systemic infection. However, further analyses are needed to understand the role of this AGO member and confirm its role in plant antiviral response. At this time, the link between AGO3 and the plant–microorganism interaction is speculative.

AGO7 is involved in the biogenesis and actions of trans-acting small interference RNAs (tasiRNAs, also called phasiRNAs), which are plant-specific endogenous siRNAs derived from long double-stranded RNA, and participate in plant development (Adenot et al., 2006). AGO7 also plays a critical role in the regulation of both plant immunity and antiviral defense (Adenot et al., 2006; Carbonell and

TABLE 2 | MiRNAs and Argonaute (AGO) proteins participating in different stages of the legume–rhizobia symbiosis (LRS)

miRNA	Associated AGO protein	Target Gene	Function in LRS	Legume Species	Reference
miR172c	AGO1/5	*AP2-1; NNC1*	Root hair deformation	*Phaseolus vulgaris; Glycine max*	Wang et al., 2014; Nova-Franco et al., 2015; Reyero-Saavedra et al., 2017
miR171c	AGO1	*NSP2*	Rhizobial infection	*Lotus japonicus*	De Luis et al., 2012
miR397	AGO1	*Laccase-Like*	Rhizobial infection	*Lotus japonicus*	De Luis et al., 2012
miR390/tasiARF	AGO7	*ARF3/4*	Rhizobial infection	*Medicago truncatula*	Allen et al., 2005
miR160	AGO1	*ARF10/16/17*	Auxins level	*Glycine max*	Turner et al., 2013; Nizampatnam et al., 2015
miR166	AGO1/10	*HD-ZIPIII*	Nodule development	*Medicago truncatula*	Boualem et al., 2008
miR167	AGO1/5	*ARF8*	Nodule development	*Phaseolus vulgaris; Glycine max*	Reyero-Saavedra et al., 2017
miR169	AGO1	*NF-YA1 (HAP2-1)*	Nodule development	*Medicago truncatula*	Combier et al., 2006

Carrigton, 2015). For example, AGO7 is also essential for the generation of the bacteria-induced small RNAs called long small interfering RNAs (lsiRNAs) (Katiyar-Agarwal et al., 2007). AtlsiRNA-1 is induced by bacterial pathogens and participates in plant resistance by silencing *AtRAP*, which encodes a RAP-domain protein involved in plant defense (Katiyar-Agarwal et al., 2007). This regulatory role of AGO7 in pathogen response mechanisms could be modulated to contribute to the fine-tuning of plant bacterial resistance under LRS. In support of this, *P. vulgaris* AGO7 is upregulated upon inoculation with rhizobia deficient in the production of NFs or lipopolysaccharides (Dalla Via et al., 2015), which are symbiotic signals able to suppress the plant defense response during symbiosis (Albus et al., 2001; Scheidle et al., 2004). Besides, mutation of the *AtAGO7* homolog in *L. japonicus* and *M. truncatula* reduces rhizobial infection and nodule number compared to the corresponding wild type (Li et al., 2014; Hobecker et al., 2017). Part of this response is also possibly due to the capacity of AGO7 to generate secondary small RNAs derived from the miR390-induced degradation of the *TAS3* transcript (Allen et al., 2005). The derived tasiRNAs target the *ARF2, 3* and *4* gene transcripts. These ARF TFs control part of the auxin signaling pathway, which also plays a key role in LRS (Breakspear et al., 2014).

AGO4/6/8/9 Clade

The AGO4/6/8/9 protein clade is oriented toward transcriptional regulation by DNA methylation (Mallory and Vaucheret, 2010; Duan et al., 2015). In legumes, this clade differs from other families. In *G. max*, *L. japonicus*, *M. truncatula*, and *P. vulgaris*, AGO4 and 6 are present but not AGO8. In the case of AGO9, this protein is absent in most legumes, except in *G. max*. This loss of diversity for the AGO8/9 group in legumes is compensated by the diversification of AGO4, which displays between two and four isoforms in the genome of model legumes (Bustos-Sanmamed et al., 2013) (**Figure 2**). This specific legume pool of AGO4 isoforms is phylogenetically separated from nonlegume AGO4, suggesting specialization in legumes. Supporting this hypothesis, in *G. max, M. trucatula*, and *P. vulgaris,* at least one of the AGO4 isoforms is differentially accumulated in nodules compared to root tissues, which suggests that this AGO4 isoform might play a role in the LRS (Phytozome v. 12.1.6).

DOES RHIZOBIAL SYMBIOSIS CAUSE DAMAGE IN THE LEGUME DNA?

Several studies have reported that plant pathogens can trigger damage in the host plant DNA (e.g., DNA double-strand breaks) (Song and Bent, 2014; Hadwiger and Tanaka, 2017). Some pathogen-induced DNA damage is triggered by reactive oxygen species (ROS) (Song and Bent, 2014; Hadwiger and Tanaka, 2017). It has been demonstrated that AGO2 and AGO9 play roles in DNA repair in *A. thaliana* (Wei et al., 2012; Oliver et al., 2014). Very recently, it has been reported that *Rhizobium huautlense* produces ROS in *Caenorhabditis elegans* intestinal cells, which then leads to DNA damage (Kniazeva and Ruvkun, 2019). Interestingly, during the rhizobial infection process, the production of ROS is essential for the formation of the infection thread (Damiani et al., 2016). Despite the evidence from animal cells and the fact that symbiotic rhizobia trigger ROS production, there is no experimental evidence to suggest that rhizobial symbiosis causes DNA damage in legume hosts. However, to allow rhizobial infection of the host, nodule cells undergo genome endoreduplication, often considered a protective mechanism against DNA damage to maintain whole-genome integrity (Maroti and Kondorosi, 2014). Further investigation is needed to explore whether rhizobia can cause DNA damage in legume hosts and whether AGO proteins (i.e., AGO2 and AGO9) participate in DNA repair in the context of LRS.

PERSPECTIVES AND CONCLUSIONS

Based on the participation of many different types of sRNAs, it is clear that different members of the AGO protein family might play crucial roles in LRS (**Figure 1**). However, it is still unclear how the participation of each AGO protein occurs and how it is regulated. Hence, the new challenge will be to understand how, when, and where AGO proteins are regulated during LRS. Having this knowledge will help us develop a clear idea about the relevance of AGO proteins in rhizobial symbiosis.

AUTHOR CONTRIBUTIONS

OV-L and DF designed the concept and organization of the manuscript. OV-L and DF wrote the manuscript with the help of MI-A, MR-S, TF-G, and MS-C.

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Tiny Yet Indispensable Plant MicroRNAs Are Worth to Explore as Key Components for Combating Genotoxic Stresses

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Plants being sessile are always exposed to various stresses including biotic and abiotic stresses. Some of these stresses are genotoxic to cells causing DNA damage by forming lesions which include altered bases, cross-links, and breaking of DNA strands, which in turn hamper the genomic integrity. In order to survive through all these adverse conditions, plants have evolved different DNA repair mechanisms. As seen from the mammalian system and different human diseases, various microRNAs (miRNAs) can target the 3′-untranslated region of mRNAs that code for the proteins involved in DNA repair pathways. Since miRNAs play an important role in plant cells by regulating various metabolic pathways, it can also be possible that miRNAs play an important role in DNA repair pathways too. However, till date, only a handful of plant miRNAs have been identified to play important role in combating genotoxic stresses in plants. Limitation of information regarding involvement of miRNAs in DNA repair as well as in ROS scavenging prompted us to gather information about plant miRNAs specific for these tasks. This mini-review aims to present pertinent literature dealing with different genotoxic stresses that cause genome instability as well as plant specific responses to survive the damage. This is intertwined with the involvement of miRNAs in genotoxic stress in plants, challenges of applying miRNAs as a tool to combat DNA damage along with ways to overcome these challenges, and finally, the future prospective of these understudied aspects.

Keywords: UV radiation, genotoxic stress, microRNAs, DNA lesions, DNA damage response network

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INTRODUCTION

Plants are always subjected to various environmental stresses which cause severe DNA damage along with genotoxic stress, which in turn may reduce the development, genome stability, and crop productivity. Drought, extreme temperature stress, salt stress, oxidative stress, and damage due to UV irradiation are the abiotic stresses encountered by the plants on daily basis (Tuteja et al., 2011). Plants are also exposed to several biotic stresses through infection by bacteria, virus, pathogens, fungi, and insects (Huang et al., 2016). These genotoxic stresses cause serious damages to plant genome and put the genome integrity at risk (Tuteja et al., 2009).

There are numerous DNA-damaging agents including but not limited to bromouracil, nitrous acid, ethyl methane sulfonate, ethidium bromide like chemical mutagens, or ROS molecules, such

as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($^{\cdot}OH$), and even different types of radiations (UV rays, gamma rays, X-rays) (Tuteja et al., 2001). Chemical mutagens damage DNA by altering DNA structure, base pairing, and base structure along with frameshift mutation. UV radiation from sunlight, consisting of UV-A, UV-B, and UV-C types of radiation, causes DNA damage by producing pyrimidine photodimers including cyclobutane pyrimidine dimers (CPD) (Hu and Adar, 2017). These pyrimidine dimers inhibit transcription and replication and induce oxidative stress. ROS overproduction is toxic to plant cells causing damage to DNA, lipids, cell membranes, and proteins (Caverzan et al., 2019).

Plants have evolved strategies to withstand continuous DNA damage and to maintain genome stability. Photoreactivation is the major DNA repair pathway in which the lesions induced by UV radiation are directly reversed back to its normal form (Friedberg, 2015). Single-strand breaks, deaminated, oxidized, or alkylated bases, are repaired by base excision repair (BER) while nucleotide excision repair (NER) works to repair CPDs, although with low capacity (Ries et al., 2000). Homologous recombination (HR) and nonhomologous end-joining (NHEJ)—mediated pathways help in repairing double-strand breaks (Spampinato, 2017). Plants have also evolved mechanisms to scavenge oxidative stress-generated free radicals through different enzymatic processes involving catalases (CATs), peroxidases (POXs), and superoxide dismutases (SODs), as well as non-enzymatic processes involving ascorbic acid and secondary metabolites (Das and Roychoudhury, 2014).

microRNAs (miRNAs) are 20–24 nucleotides long, small non-coding ribonucleic acids, involved in the regulation of gene expression by interfering with various post-transcriptional processes (Yu et al., 2017). Plant miRNAs play vital roles in growth and development as well as in tolerating several types of biotic and abiotic stresses like extreme temperatures, nutrient deprivation, and salinity (Li et al., 2016). Plant miRNAs control gene expression either by cleavage of the target mRNA or through translational inhibition (Xie et al., 2015). miRNAs specifically identify targets by base complementarity and, in turn cleave, translationally repress or destabilize the target mRNAs (Moro et al., 2018). Perfect base pairing of miRNA with target mRNA leads to the cleavage of the targets, whereas the imperfect binding results in translational repression of the target mRNAs (Djami-Tchatchou et al., 2017).

A highly controlled regulation is required to maintain the DNA damage response (DDR) network and ROS scavenging mechanisms to combat genotoxic stresses in plant cells. It is yet to explore whether plant miRNAs play substantial roles in regulating the expression of the genes that are directly or indirectly involved in genotoxic stresses. The fact that only a handful of studies have considered the involvement of miRNAs in DDR and ROS scavenging prompted us to gather information about plant miRNAs specific for these functions. However, we have faced several hurdles in this task due to the unavailability of miRNAs and direct involvement of their corresponding targets within the DDR network. Based on the available information, we have discussed about different genotoxic stresses in plants, role of plant miRNAs in combating genotoxic stresses, hurdles in applying miRNAs as a tool to combat genotoxic stresses in plants, and ways to overcome these problems. The gathered information will be helpful for future practical application of miRNAs as potential tools to secure and stabilize crop yield in view of the continuous climatic changes.

GENOTOXIC STRESSES LEADING TO INSTABILITY IN PLANT GENOME

There are numerous DNA-damaging processes continuously threatening the integrity of the plant genome, including various chemical mutagens and UV radiation, the latest being amongst the most hazardous. These types of stresses generate various DNA lesions that includes altered, missing, and mismatched bases; single- or double-strand breaks; insertion or deletion of bases; pyrimidine dimers; and cross-linked DNA strands, which are genotoxic to plant cells (Tuteja et al., 2001). These damages in turn inhibit transcriptional and translational processes which ultimately affect plant growth and crop yield.

DNA Damage Due to the Production of Free Radicals

Plant cells get damaged by the excess production of ROS which includes free radicals like H_2O_2, O_2^-, and.OH (Inzé and Montagu, 1995; Sharma et al., 2012). Although O_2^- and H_2O_2 can damage the DNA, these two radicals are very unstable and can easily be removed from the system in the absence of metal catalysts (Tuteja et al., 2009). Conversion of O_2^- and H_2O_2 to.OH is catalyzed by metals, and.OH is the major source of toxicity in the cells as it reacts with almost all the cellular macromolecules including DNA (Sharma et al., 2012). Lipid peroxidation can also induce the production of ROS which leads to cross-linking of DNA and proteins; hence, they are toxic and mutagenic for cells (Gaschler and Stockwell, 2017). The reactive electrophiles are responsible for the production of various DNA adducts, namely, propano adducts, adducts of acrolein, and crotonaldehyde. The 4-hydroxynonenal compound is the most genotoxic whereas malondialdehyde (MDA) is considered the most mutagenic products of lipid peroxidation (Łuczaj and Skrzydlewska, 2003).

DNA Damage Induced by UV Radiation

UV radiation plays an important role in damaging plant genome stability by producing pyrimidine hydrates as a result of oxidative damage and cross-links between DNA and/or protein, and in turn, inhibits plant growth and development (Gill et al., 2015). UV-B, being the most harmful form of UV radiation, is responsible for the production of DNA lesions like CPD and pyrimidine (6–4) pyrimidinone adducts (6–4 PPs) (Ries et al., 2000; Law et al., 2013). CPDs are found to block transcribing complexes, which in turn is responsible for the alteration of gene expression patterns. In addition to CPD-mediated damage, UV-B also induces delay of G1-to-S phase transition within the plant cell cycle (Jiang et al., 2011). UV-C induces both single-stranded breaks and double-stranded breaks in *Arabidopsis* (Abas et al., 2007). Oxidative DNA damages are also found to be responsible

for UV-associated mutagenicity and instability of plant genome (Manova and Gruszka, 2015).

PLANT CELLULAR RESPONSES TO DNA DAMAGE

Plants respond to DNA damage by activating a complex DDR network consisting of mechanisms like DNA repair, cell cycle arrest, and apoptosis (Yoshiyama et al., 2013). Different types of DNA repair mechanisms like photoreactivation, BER, NER, mismatch repair (MMR), and double-strand break (DSB) repair gets activated in plants in response to DNA damage (Kimura and Sakaguchi, 2006). It is found that large numbers of protein components are involved in these repair mechanisms, a handful of which being potential target of miRNAs. The involvements of some of these protein components in the repair mechanisms are briefly discussed.

In the photoreactivation-mediated DNA repair mechanism, thymine dimer structures are found to be cleaved by CPD lyase or (6–4) photolyase (Waterworth et al., 2002). Photolyases bind specifically to the damaged site of double-stranded DNA in a light independent manner, although it gets activated through UV-A for correction of the lesions. This is followed by the splitting of the covalent bonds of the dimers in an error-free manner (Manova and Gruszka, 2015).

Within BER, apurinic/apyrimidinic sites are found to be recognized by lesion-specific DNA glycosylases which cleave the N-glycosidic bond following the removal of the affected base and the generation of abasic sites in plants (Manova and Gruszka, 2015). In *Arabidopsis*, carrot, and rice, several DNA glycosylases were identified—as a couple of examples, 3-methyladenine-DNA glycosylase (MAG), formamido-pyrimidine-DNA glycosylase (FPG) 8-oxoG DNA-glycosylases (OGG), uracil-DNA glycosylase (UNG), and DNA glycosylase/lyase DNG701 (Santerre and Britt, 1994; Dany and Tissier, 2001; Talpaert-Borlé and Liuzzi, 2005; La et al., 2011). NER recognizes and repairs several types of DNA lesions induced by UV-rays and other mutagens, and the process was extensively studied in *Arabidopsis thaliana*. *Xeroderma pigmentosum* complementation of group C (XP-C)/AtRAD4 recognizes DNA damage. This is followed by the unwinding of DNA containing damaged portion by the transcription factor IIH (TFIIH), including AtXP-D. Damaged oligonucleotides get excised by AtXP-F or other *Arabidopsis* homologs (e.g., AtERCC1). The excised gap is filled through the activity of proliferating cell nuclear antigen (PCNA) and replication factor C (RFC)–mediated DNA synthesis. Finally, DNA ligase I joins the DNA strands (Xu et al., 1998; Ishibashi et al., 2003; Kimura and Sakaguchi, 2006; Molinier et al., 2008; Liu et al., 2012).

Within MMR, homologs of MutS (MSH) recognize the mismatch generated by the incorporation of incorrect bases by DNA polymerase. This is followed by generation of nicks through the activity of MutL (MLH) homologues and the successive steps of the repair system (Dion et al., 2007; Lario et al., 2015).

DSBs are repaired in plants by HR- and NHEJ-mediated pathways (Puchta, 2005). In *Arabidopsis* and rice, many components of DSB repair mechanisms have been identified—for example, AtRad51, AtRadA, AtRad50, OsRadA, AtMre11, AtKu70, AtKu80, *Arabidopsis* DNA ligase IV, AtXRCC4, AtXP-F, and AtERCC1 (Manova and Gruszka, 2015). Additionally, it has been discovered that Ataxia telangiectasia–mutated (ATM) and ATM-Rad3-related (ATR) proteins play important roles in DNA repair. Checkpoint kinases (CHK), including Chk1 and Chk2, work downstream of ATM and ATR proteins, where activated ATR initiates G-2 phase arrest by phosphorylating CHK1. This in turn is responsible for DDR-induced transcriptional repression (Culligan et al., 2004).

INVOLVEMENT OF PLANT MIRNAS IN GENOTOXIC STRESS TOLERANCE

Till date, a handful of plant miRNAs with active role in combating genotoxic stresses have been identified in plants. Although the number is considerably low, we can divide these miRNAs into two categories. One set of miRNAs is involved in tolerating oxidative stress, while the other type of miRNAs may play an active part in DNA repair. A large set of enzymes acting as the key regulators of both ROS scavenging and DNA repair mechanism may be targeted by both these types of miRNAs. We have tabulated plant miRNAs found to target various enzymes involved in ROS scavenging and DDR network in **Table 1**. A schematic depiction of the involvement of miRNAs in these two processes is also shown in **Figure 1**.

miRNA Involvement in ROS Scavenging

SOD, a metal containing enzyme, is one of the most important enzymes for the removal of ROS produced by oxidative stress (Das and Roychoudhury, 2014). There are different types of SODs, including mitochondrial MnSOD, cytosolic and chloroplastic Cu/ZnSOD, and chloroplastic FeSOD (Van Camp et al., 1990; Bowler et al., 1994; Fukai and Ushio-Fukai, 2011). The complete regulatory mechanism of CSD1 and CSD2 is unknown, but recent studies have proved that miRNAs are

TABLE 1 | Involvement of miRNAs in targeting various enzymes involved in ROS scavenging as well as DDR network in plants.

Enzymes	miRNAs	Plant
Superoxide dismutase	miR398	*Arabidopsis* (Sunkar et al., 2006) Rice (Li et al., 2010) Wheat (Qiu et al., 2018), (Biselli et al., 2018) Grapevine (Leng et al., 2017) Barley (Xu et al., 2014b) Common bean (De la Rosa et al., 2019)
Photolyase	miR838b	*Brassica rapa* (Hajieghrari et al., 2017)
Helicases	miR414, miR408, miR164e	Rice (Macovei and Tuteja, 2013)
TCP gene	miR319	*Arabidopsis* (Koyama et al., 2017)

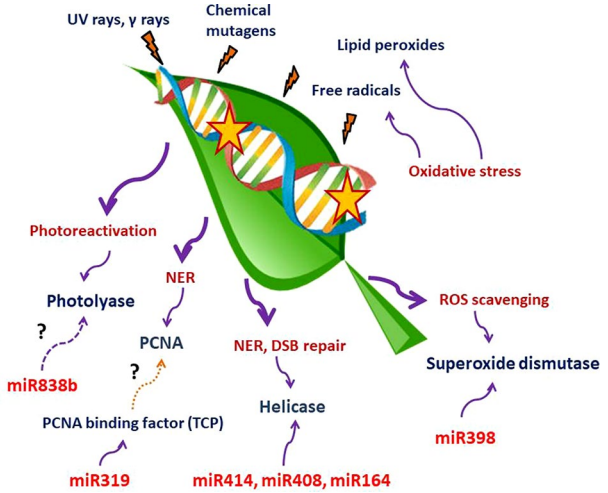

FIGURE 1 | Schematic representation of miRNAs involvement in ROS scavenging and DNA repair mechanisms. UV and γ-rays, chemical mutagens, free radicals, and lipid peroxidation produced due to oxidative stress are among the most important DNA damage–causing agents. miR398, miR319, miR838b, miR414, miR408, and miR164 have been shown to target components involved in the abovementioned processes. The question mark indicates that more studies are required to establish the link with the adjacent pathways. There is no direct evidence of miR319 targeting TCPs that bind to the PCNA promoter (brown dotted line). Targeting of photolyase by miR838b through computational prediction needs experimental confirmation (magenta dashed line).

having a role in this. Sunkar et al. (2006) have shown that the *Arabidopsis* ath-miR398 has a role in the regulation of CSD1 and CSD2. They found that downregulation of miR398 expression is important for the accumulation of CSD1/2 mRNA. While experimenting with different tissues, the same group of scientists have found that tissues like cauline leaves, stems, and roots (having high levels of miR398) showed lower expression of CSD1/2 mRNAs, whereas tissues like old rosette, leaves, and inflorescence (having low level of the same miRNAs) showed higher levels of CSD1/2 expression. Additionally, in rice, it was evidenced that miR159 targets Cu/ZnSOD (At5g18100-CSD3) (Li et al., 2010). Another group of scientists has shown that CSD is a potential targets of miR398 in wheat seedlings exposed to drought (Qiu et al., 2018). Cytosolic CSD1/2 was found to be targeted by miR398 in wheat in response to the fungal attack by *Fusarium graminearum* which causes *Fusarium* head blight disease (Biselli et al., 2018). Cytosolic CSD1 and chloroplastic CSD2 are potential targets of Vv-miR398 in grapevines (Leng et al., 2017). The Vv-miR398 family is highly conserved, and three loci, namely, miR398a (located on the chromosome no. 1), miR398b, and miR398c (located on the chromosome no. 6) encode the *MIR* gene in grapevine. In barley, hvu-miR398 was found to be negatively regulated by Mildew resistance locus a (Mla) and Mla resistance1 (rom1), and the overexpression of miR398 is responsible for the reduction in CSD1 (Xu et al., 2014b). In another experiment carried out in *Phaseolus vulgaris*, it has been shown that repression of miR398 leads to upregulation of CSD1 expression in case of water deficit (De la Rosa et al., 2019).

miRNA Involvement in DNA Repair Mechanism

When considering the direct implication of miRNAs in plant DNA repair mechanisms, some helicases, important enzymes involved in both NER and DSB repairs, have been shown to be targeted by miR164, miR408, and miR414 in rice (Macovei and Tuteja, 2013). Several studies have addressed putative miRNA targeting mRNAs of genes involved in DDR by *in silico* analyses. For example, using psRNATarget server, a computational tool to predict miRNA targets, it was found that *Brassica rapa* miR838b putatively targets the photolyase mRNA (Hajieghrari et al., 2017). However, this prediction remains to be confirmed experimentally.

As an example of indirect involvement of miRNAs in DDR downstream processes, several studies established the contribution of miR319 to the regulation of TCP (teosinte-branched1/*Cincinnata*/proliferating cell factor) transcription factors, playing direct roles in leaf development (Danisman, 2016; Koyama et al., 2017; Bresso et al., 2018). It has been shown that two transcription factors, namely, PCF1 and PCF2, containing the non-canonical basic helix–loop–helix motif TCP domain, can regulate the transcription of PCNA (proliferating cell nuclear antigen) gene, an important component of the DDR network (Danisman, 2016; Nicolas and Cubas, 2016). Among the 24 members of *Arabidopsis* TCP family, miR319 targets the transcripts of *TCP2, TCP3, TCP4, TCP10,* and *TCP24* genes (Koyama et al., 2017). However, till date, there is no direct evidence showing that miR319 targeting TCPs can directly bind to the PCNA promoter.

CHALLENGES RELATED TO MIRNA APPLICATIONS IN COMBATING GENOTOXIC STRESSES IN PLANTS

Hurdle I: Although many miRNAs have been found to be involved in the DDR network in human cells, very few miRNAs are found to be involved in DNA repair in plants. DNA repair processes are well characterized in mammalian systems; in contrast, very few studies are done in plants, and these are mainly limited to *Arabidopsis* and rice (Ueda and Nakamura, 2011; Macovei and Tuteja, 2013; Manova and Gruszka, 2015).

Hurdle II: There is absence of focused research on targeting genes involved in DNA repair even when considering miRNAs that are extensively studied in plants in relation to stress response; however, information about targets of miRNAs that are specifically involved in combating genotoxic stresses in plants is much more limited. Differently, many studies are being performed to find the targets of miRNAs in DDR in human diseases, including cancer studies (Hu and Gatti, 2011; He et al., 2016).

HOW TO OVERCOME THE CHALLENGES?

Identification of more plant miRNAs involved in DNA repair and ROS scavenging is one of the ways to overcome the challenge. In this regard, RNA sequencing of plants exposed to genotoxic stresses will lead to identification of new miRNAs specifically associated with genotoxic stresses. Once a substantial number of miRNAs are being identified in plants, their targets need to be validated. For target identification, bioinformatics approaches, along with experimental validation of the same by 5' rapid amplification of cDNA ends (RACE), parallel analysis of RNA ends (PARE), degradome-seq, or genome-wide mapping of uncapped transcripts, can be of great help. References from miRNAs involved in DDR in human diseases can be taken, and extensive studies must be performed to find out the role of plant miRNAs in targeting the homolog proteins involved in DNA repair in plant species.

Several human diseases are found to be associated with miRNA-dependent regulation of DNA repair pathways. For example, RAD23 and CDK7 are two important enzymes in NER pathway. miR-494 was found to target RAD23 homolog B (Comegna et al., 2014), while CDK7 is targeted by miR-210 (Abdullah et al., 2016). Human miR-103a-2-5p and miR-585-5p target poly ADP-ribose polymerase (PARP), an important BER enzyme (Dluzen et al., 2017). Human miR-422a base pairs with MLH1 3′-untranslated region and suppresses the expression of the same which in turn downregulate MutLα, a key protein of the MMR (Mao et al., 2012). In colorectal cancer cells, miR-7 targets XRCC2, a core protein involved in HR (Xu et al., 2014a). In cancer cell lines, RAD51 and BRCA1/2 (breast/ovarian cancer susceptibility gene products) are key proteins responsible for catalyzing HR, and both the proteins are potential target of miR-103 and miR-107 (Huang et al., 2013). BRCA1/2 is also found to be targeted by miR-15/107/182 in breast cancer (Petrovic et al., 2017). MSH2, another essential MMR component, is downregulated by miR-21 in human (Valeri et al., 2010). Homologs of all the human RAD23, CDK7, PARP, MSH, MLH, XRCC2, RAD51, and BRCA1/2 are present in rice. However, there is no information available about potential miRNAs that target these mRNAs. While studying NHEJ repair in lung-cancer cell line, Yan et al. (2010) reported that miR-101 targets 3′- UTR of DNA-PKcs, a core component of NHEJ. In the same study, it has been proved that ATM, another key protein of HR-mediated repair, is a target of miR-101. Even tough plant homologues of ATM are reported in *Arabidopsis* and rice, no miRNA associated to their sequences was identified. Hence, it is noteworthy to mention that, even though it is well-established, several human genes involved in DNA repair are targeted by miRNAs, and some of their homologs are also reported in plants. No information is available about miRNAs targeting these genes in plants. With the advancement of genome annotation techniques and the availability of published and draft genomes, miRNAs can be searched firstly through bioinformatics approach. Once detected, these miRNAs can be experimentally verified for differential regulation of the target miRNAs associated with genotoxic stresses.

CONCLUSION AND FUTURE PERSPECTIVES

DNA repair is a very important mechanism that allows plant cells to overcome genotoxic stresses and to maintain genome integrity. Impaired DNA repair mechanisms are the reason for plant slow growth and development, which in turn causes the reduction in crop production. In recent years, miRNAs have been identified as potentially novel and vital regulators of biological processes, including developmental processes and diseases. Considering their importance, it is essential to know more about miRNAs and their targets associated with DNA repair mechanism in plants. Once we find out the specific role of miRNAs and their targets in DNA repair and ROS scavenging, we could engineer them with genome-editing technologies like CRISPR-Cas, and hence aiming to combat a great number of genotoxic stresses.

AUTHOR CONTRIBUTIONS

MC wrote the manuscript. JB conceived the study and participated in its coordination.

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A Bioinformatics Approach to Explore MicroRNAs as Tools to Bridge Pathways Between Plants and Animals. Is DNA Damage Response (DDR) a Potential Target Process?

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MicroRNAs, highly-conserved small RNAs, act as key regulators of many biological functions in both plants and animals by post-transcriptionally regulating gene expression through interactions with their target mRNAs. The microRNA research is a dynamic field, in which new and unconventional aspects are emerging alongside well-established roles in development and stress adaptation. A recent hypothesis states that miRNAs can be transferred from one species to another and potentially target genes across distant species. Here, we propose to look into the *trans*-kingdom potential of miRNAs as a tool to bridge conserved pathways between plant and human cells. To this aim, a novel multi-faceted bioinformatic analysis pipeline was developed, enabling the investigation of common biological processes and genes targeted in plant and human transcriptome by a set of publicly available *Medicago truncatula* miRNAs. Multiple datasets, including miRNA, gene, transcript and protein sequences, expression profiles and genetic interactions, were used. Three different strategies were employed, namely a network-based pipeline, an alignment-based pipeline, and a *M. truncatula* network reconstruction approach, to study functional modules and to evaluate gene/protein similarities among miRNA targets. The results were compared in order to find common features, e.g., microRNAs targeting similar processes. Biological processes like exocytosis and response to viruses were common denominators in the investigated species. Since the involvement of miRNAs in the regulation of DNA damage response (DDR)-associated pathways is barely explored, especially in the plant kingdom, a special attention is given to this aspect. Hereby, miRNAs predicted to target genes involved in DNA repair, recombination and replication, chromatin remodeling, cell cycle and cell death were identified in both plants and humans, paving the way for future interdisciplinary advancements.

Keywords: bioinformatics, DNA damage response, microRNA, networks, *trans*-kingdom

INTRODUCTION

The classical definition describes microRNAs (miRNAs) as small non-coding, single-stranded molecules that bind to mRNA by sequence complementarity and inhibit gene expression through posttranscriptional regulation (Bartel, 2004; Pasquinelli, 2012). By doing so, miRNAs are involved in many cellular and developmental processes, acting as master-regulators of gene expression. It is well-known that miRNAs are evolutionarily conserved in eukaryotes, although some differences exist between animals and plants, mainly related to their biogenesis and target recognition mechanism (see reviews by Millar and Waterhouse, 2005; Moran et al., 2017). In plants microRNAs are produced in nucleus and exported to cytoplasm, whereas in animals pri-microRNA and pre-microRNA are produced in the nucleus while the microRNA/microRNA* are produced in the cytoplasm. Both plant and animal miRNAs associate with the RISC complex, indispensable for miRNA activity, in the cytoplasm. In animals, pri-miRNAs are first cleaved by Drosha RNase III while in plants this is carried out by Dicer-like (DCL)1. Plant miRNAs have a 2′-O-methylation on the 3′-terminal nucleotide which is not present in animal miRNAs. Considering the target recognition mechanisms, in plants this is based on near-perfect or perfect sequence complementarity (leading mostly to mRNA decay), whereas in animals the sequence complementarity is imperfect, mostly based on the 'seed rule' (base pairing to the 5′ end of miRNAs, especially nucleotides 2–7) (Lewis et al., 2005).

Emerging research proposes a novel and controversial hypothesis indicating that miRNAs can be transferred from one species to another and potentially target genes across distant species. This concept has been developed starting from evidence showing that small RNAs can move from cell to cell (Molnar et al., 2010) and can act in gene silencing (RNA interference) across species (see reviews by Han and Luan, 2015; Weiberg et al., 2015). While the transfer of miRNAs from plants or humans/animals to their pathogens (Valadi et al., 2007; LaMonte et al., 2012; Buck et al., 2014) is less disputed, the situation gets more complicated when addressing the plant miRNA transfer to humans. This is due to several open questions and contrasting results regarding plant miRNA stability, abundance, mode of action, and validation of potential targets in human cells (Dickinson et al., 2013; Tosar et al., 2014; Micó et al., 2016; Cavallini et al., 2018). The first direct indication that ingested plant miRNAs, derived from food, can target genes in a cross-kingdom fashion had been provided by Zhang et al. (2012). The authors showed that a rice miRNA (osa-miR168a) stably exists in the sera and tissues of animals and humans and it specifically targets the liver low-density lipoprotein (LDL) receptor adapter protein 1 (LDLRAP1), decreasing the removal of LDL from plasma. Briefly, this research proposes that plant miRNAs are released from destroyed cells (during mechanical mastication) and transferred to the intestinal epithelial cells, where they could be incorporated into vesicles (exosomes or microvesicles) and enter the circulatory system to be delivered to targeted cells. Plant miRNAs can resist the activity of digestive enzymes and low pH throughout the gastrointestinal tract due to their methylation and high GC content (Zhang et al., 2012; Philip et al., 2015; Zhou et al., 2015). Moreover, immunoprecipitation experiments with anti-AGO2 antibodies have shown that miR168a associates with AGO2 in Caco-2 cells, thus enabling miRNAs' function (Zhang et al., 2012). This was also confirmed in another study where immunoprecipitation data revealed that honeysuckle (*Lonicera japonica*) miR2911 associated with the AGO2 complex in microvesicles (Zhou et al., 2015). In this study, miR2911 has been demonstrated to be resistant to processing and proposed to target genes involved in the resistance to viral influenza. Hence, resistant exogenous plant miRNAs may regulate multiple target genes based on sequence complementarity, similarly to how endogenous miRNAs act (Liu et al., 2017). This concept expands the known types of miRNA functions to key natural bioactive compounds with potential health promoting benefits (depending on the mRNA target). So far, compelling evidence has demonstrated that plant miRNAs are present in human/animal plasma and these miRNAs usually belong to evolutionary conserved families (Vaucheret and Chupeau, 2012; Zhang et al., 2012; Liang et al., 2014; Yang et al., 2015a; Yang et al., 2015b; Cavalieri et al., 2016). Plant miRNAs not only from edible plant species (rice, cabbage, broccoli, watermelon, soybean, strawberry, olive) but also from model (Arabidopsis, poplar) and medicinal plants (Moringa, honeysuckle, turmeric, ginger) had been evaluated for their potential *trans*-kingdom transfer (Zhang et al., 2012; Liang et al., 2014; Zhou et al., 2015; Cavalieri et al., 2016; Chin et al., 2016; Pirrò et al., 2016; Liu et al., 2017; Sharma et al., 2017; Minutolo et al., 2018).

Aside from the biomedical interest, miRNAs *trans*-kingdom interactions can be useful to better understand evolutionary distant conserved pathways. Some examples of preserved pathways between plants and animals include the innate immune signaling pathways (Ausubel, 2005), programmed cell death (PCD)-related pathways (Godbole et al., 2003; Lord and Gunawardena, 2012), some basic functions (e.g. Ca^{2+}ATPase, Ca^{2+}/Na^{+}-K^{+} ion exchanger) of calcium signaling pathway (Nagata et al., 2004), and the DNA damage response (DDR) (Yoshiyama et al., 2013; Nikitaki et al., 2018). Among these, DDR is defined as a complex signal-transduction pathway consisting of DNA damage sensors, signal transducers, mediators, and effectors which in turn activate a series of events (e.g. phosphorylation cascades) that lead to the regulation of downstream processes (e.g. cell cycle checkpoint, DNA repair), common between the plant and animal kingdoms (Yoshiyama et al., 2013). The involvement of miRNAs in the regulation of DDR players is quite recent and insufficiently explored, especially within the plant kingdom. Conversely, studies in human cells have already shown that miRNAs are involved in the regulation of DDR-associated genes and their activity is intricately weaved with traditional elements such as ATM (ataxia-telangiectasia mutated) and p53 (Kato et al., 2009; Landau and Slack, 2011; Wan et al., 2011). In plants, some miRNAs (e.g. osa-miR414, osa-miR164e, and osa-miR408), have been demonstrated to target specific helicases with roles in DNA repair, recombination, replication and translation initiation (Macovei and Tuteja, 2012; Macovei and Tuteja, 2013).

The current work aims to investigate the *in silico trans*-kingdom valence of plant miRNAs as a potential tool to

bridge conserved pathways between plant and human cells, inquiring their implication in DDR. To do so, a multi-faceted bioinformatics approach was developed by combining and evaluating different data- or knowledge-driven resources and tools. The model legume *Medicago truncatula* (barrel medic) has been chosen as target for this analysis because of its potential medicinal properties (high content in saponins) (Tava et al., 2011), sequenced genome and availability of different databases (Goodstein et al., 2012), as well as its conserved synteny among legumes (Gujaria-Verma et al., 2014; Lee et al., 2017) which can offer the possibility of translational applications to other economically relevant species. Moreover, in view of promoting future sustainable agriculture practices and food security, microgreens, defined as seedlings harvested when the first leaves appear, are gaining momentum as novel functional food sources with high nutritional content and health-promoting benefits (Choe et al., 2018). In this context, legume species previously used only as fodder, like *Trifolium* spp., *Medicago* spp. and *Astragalus* spp., are now being proposed as microgreens for human consumption since they had been demonstrated to contain high protein and phytochemical contents as well as low levels of carbohydrates (Butkutė et al., 2018). Hence, starting from a collection of *M. truncatula* miRNAs, we retrieved candidate targets in plant and human transcriptomic datasets and analyzed them with different strategies: *(1)* a gene network-based strategy was used to compare the targeted biological processes in plant and human, using an *Arabidopsis thaliana* homology-based approach for plant network reconstruction; *(2)* an alignment-based strategy was used to identify nucleotide and protein similarities between *M. truncatula* and *Homo sapiens* putative targets; *(3)* another network-based strategy was carried out by using a *de novo* reconstructed *M. truncatula* gene network to further assess the common biological processes targeted in human and barrel medic. All the above-mentioned strategies have been used for the common purpose of identifying shared features (e.g. microRNAs targeting similar processes) between these distantly related organisms.

MATERIALS AND METHODS

The workflow followed in this study is illustrated in **Figure 1** and its parts are discussed below. Three different strategies were employed, namely a network-based pipeline, an alignment-based pipeline, and a *M. truncatula* network reconstruction approach.

Datasets

The list of *M. truncatula* miRNAs was retrieved from miRBase (Kozomara et al., 2019) and included 756 sequences, among which 426 were unique. The human 3′ UTRome sequence dataset was retrieved from the psRNATarget tool web site (Dai et al., 2018) and included 21,233 sequences, among which 18,167 were relative to unique genes. The *M. truncatula* transcript dataset (Mt4.0 v1) was retrieved from the psRNATarget tool web site and included 62,319 transcripts, corresponding to 50,894 unique genes.

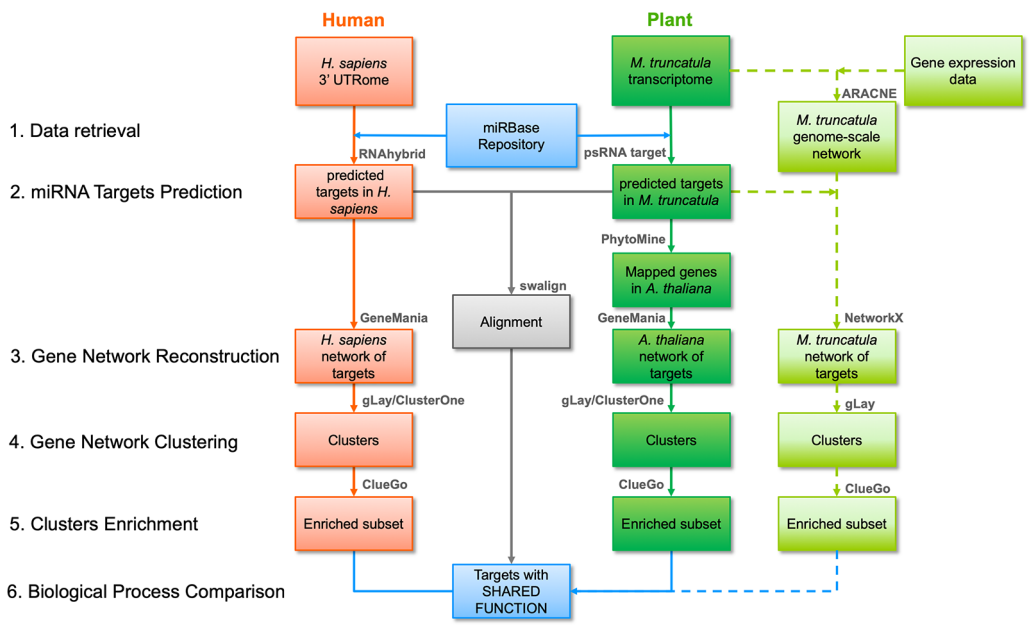

FIGURE 1 | Bioinformatic workflow followed in this work including network- and alignment-based analysis pipelines. The main steps of the network-based pipeline are numbered on the left, at the same level as the pipeline blocks indicating input and output of each step. Red and dark green blocks indicate human and plant inputs/outputs, respectively, and data flow is reported with arrows. The main software tools or functions (detailed in the main text) are summarized above each block. Light green blocks indicate inputs/outputs for the *Medicago truncatula* network-based pipeline, also including genome-scale network construction, and its data flow is reported as dashed arrows. The outputs of the alignment-based pipeline are reported as a single grey block indicating the sequences with significant similarity after alignment. Blue blocks indicate the initial and final data for human and plant in both analysis pipelines.

The gene sequences and the related protein sequences of the predicted targets were retrieved from the NCBI RefSeq database (for human targets) (O'Leary et al., 2016), and from the annotated coding sequence and protein datasets from the *M. truncatula* Genome Database (for plant) (Krishnakumar et al., 2014).

Six microarray datasets from the ArrayExpress (Kolesnikov et al., 2015) repository were used: E-MEXP-1097 (Benedito et al., 2008), E-MEXP-3719 (Verdier et al., 2013), E-MEXP-2883 (Tang, 2014), E-MEXP-3190 (Uppalapati et al., 2012), E-MEXP-3909 (Wang et al., 2016), and E-GEOD-43354 (Limpens et al., 2014). These amounted to a total of 117 raw expression samples (in CEL format) that were used for *M. truncatula* co-expression network reconstruction. The dataset samples measured under perturbed conditions (e.g. salt or drought stress, infections) were excluded. All the considered experiments were conducted on the same microarray platform (Affymetrix GeneChip Medicago Genome array), thereby avoiding genome annotation biases.

miRNA Target Prediction

The psRNATarget (Dai et al., 2018) and RNAhybrid (Kruger and Rehmsmeier, 2006) online tools, specific for miRNA target prediction in plants and mammalians, respectively, were used. The list of *M. truncatula* unique miRNAs was used as input for both tools, together with the *M. truncatula* transcript dataset or the human 3′ UTRome (unless differently indicated). The 3′ UTR region was chosen under the assumption that plant miRNAs can regulate human targets in the same manner as endogenous human miRNAs (Bartel, 2004). This assumption is consistent with a number of recent bioinformatics works, which were in some cases further validated, leading to experimental evidence of cross-kingdom regulation (Shu et al., 2015; Chin et al., 2016; Zhang et al., 2016a; Hou et al., 2018; Zhao et al., 2018a). Despite this commonly performed assumption, it is worth noting that no *golden standard* exists for plant miRNA target prediction in a cross-kingdom context (Lukasik et al., 2018). A small number of other works additionally considered 5′ UTR and/or coding sequences as potential target regions (Liu et al., 2017; Lukasik et al., 2018; Mal et al., 2018). This was motivated by studies in which different transcript regions have been reported as non-3′ UTR targets for both endogenous and cross-kingdom regulations (Li et al., 2018; Wang et al., 2018). With the availability of additional validation studies and models for cross-kingdom regulation, this gap will be filled. Importantly, the proposed workflow can be easily adapted by changing the target sequences files.

The parameters of the two target prediction tools were set to obtain a balanced number of network nodes (about 700 for *A. thaliana* and *H. sapiens*) in the network-based pipeline, and of unique target transcripts (about 1,700 for *M. truncatula* and *H. sapiens*) in the alignment-based pipeline. A highly specific hybridization in seed region, typically occurring in plants, was set in psRNATarget, which was used to find plant target genes for network-based pipeline with the following parameters: number of top targets = 50, Expectation = 2.5, Penalty for G:U pair = 0.5, Penalty for other mismatches = 1, Extra weight in seed region = 1.5, Seed region = 2–13 nucleotides, Mismatches allowed in seed region = 0, HSP size = 19. The list of targets for the alignment-based pipeline was obtained *via* the same parameters as above except the number of top targets which was set to 15. The predicted target list from RNAhybrid was filtered by tuning the sole algorithm parameter that is Minimum Free Energy (MFE), whose threshold was set to −36.5 kcal/mol, while for the alignment-based pipeline it was −34.7 kcal/mol. In both cases, a maximum of 50 targets per miRNA was considered (Zhang et al., 2016a).

Network-Based Pipeline

The lists of predicted targets were used to construct plant and human target networks using GeneMania, (Warde-Farley et al., 2010), and considering all the genetic and co-expression interactions available within the tool. Since GeneMania does not contain *M. truncatula* among the available organisms, the following procedure was used to construct a genetic interaction/co-expression network of *A. thaliana*, by mapping the homologous genes of the *M. truncatula* predicted targets list. The Phytomine tool (Goodstein et al., 2012) of the Phytozome portal (JGI) was used to obtain a mapping from the *M. truncatula* target genes to *A. thaliana* genes, based on homology. Correspondences between the species were considered with a relative threshold similarity above 85%.

Human and plant networks were imported and analyzed using Cytoscape (v.3.7.1) (Shannon et al., 2003) and its applications. Clustering was carried out using the gLay (Su et al., 2010) and ClusterOne (Nepusz et al., 2012) algorithms, considering the networks as undirected and unweighted. ClusterOne was used with the following parameters: minimum size = 50, minimum density = 0.25, unweighted edges, node penalty = 2, haircut threshold = 0, merging method = Multi-pass, Jaccard similarity, overlap threshold = 0.15, seeding method from unused nodes. The gLay algorithm does not have free parameters. For each cluster, enrichment analysis was carried out using ClueGO (Bindea et al., 2009) to find statistically over-represented Gene Ontology (GO) terms in the Biological Process (BP) category, using a right-tail test with the Benjamini–Hochberg correction for multiple testing, and a 75% detail level. GO terms were considered for further analysis if they had p-value < 0.05 and if at least one of the related genes was present in the original target gene list (since GeneMania includes interactor genes not belonging to the input target list). The analysis procedure followed in this network-based pipeline is summarized in **Figure 2**.

Alignment-Based Pipeline

For each miRNA, the nucleotide coding sequence and protein sequence of the predicted transcript targets found in *M. truncatula* and *H. sapiens* were compared *via* sequence alignment. A custom MATLAB R2018a (MathWorks, Natick, MA, USA) script was programmed to automatically carry out this analysis and to evaluate the statistical significance of each comparison. The Smith–Waterman method (Smith and Waterman, 1981) was used to perform local alignment *via* the *swalign* function and get the optimal alignment score (in bits) as output. A random permutation-based statistical analysis was adopted to evaluate the significance of each alignment and to obtain a sequence

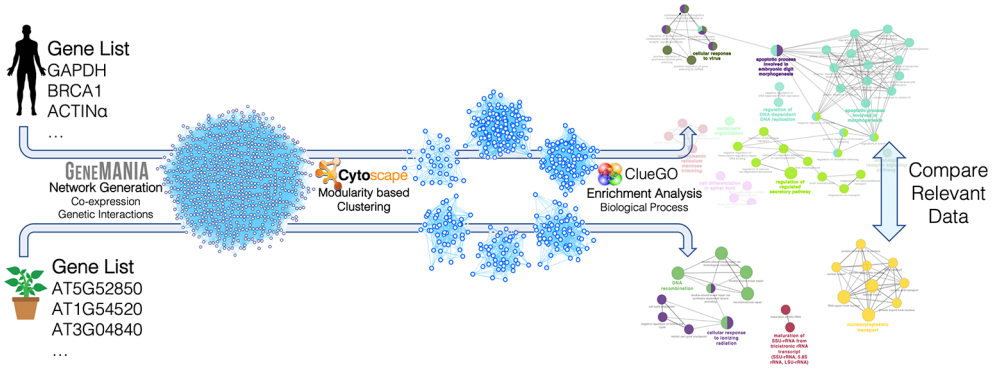

FIGURE 2 | Construction and analysis steps of the miRNA target networks for *Arabidopsis thaliana* and *Homo sapiens*. The lists of miRNA target genes were used to construct genetic interaction/co-expression networks with GeneMania. The resulting networks were analyzed with Cytoscape and its applications. In particular, clustering was carried out with two different modularity-based methods (gLay and ClusterOne) and enrichment analysis was carried out with the ClueGO app to find enriched biological processes in each cluster. The resulting processes found for the two organisms were finally compared, taking into account the related target genes and miRNAs.

length-independent scoring value (*p*-value) (Teng et al., 2014; Tiengo et al., 2015). Specifically, for each sequence comparison, 200 random permutations were constructed for the human nucleotide/protein sequence and an alignment was performed for each randomization. The resulting distribution of bits scores was used to obtain the final *p*-value as the number of alignments giving a bits score higher than the original one, divided by the number of randomizations. Low *p*-values correspond to statistically significant alignments with a considered threshold of 0.05. The analysis procedure followed in this alignment-based pipeline is summarized in **Figure 3**.

Reconstruction of *M. truncatula* Co-Expression Network

Raw expression values were globally normalized using the Robust Multichip Average (RMA) method (Irizarry et al., 2003), and then annotated using the MedtrA17_4.0 *M. truncatula* reference genome assembly. Array probes mapping the same gene were median-averaged and those lacking functional annotation were discarded. Co-expression analysis of the obtained expression panel was performed *via* ARACNE (Margolin et al., 2006) by: *(i)* building the Mutual Information Matrix using the Spearman correlation, and then *(ii)* pruning the obtained interactions among all possible gene triplets with null mutual information. All the analyses were performed in the R environment, using the *limma* (Ritchie et al., 2015) and the *biomaRt* (Durinck et al., 2009) packages for the expression data preparation, and the *minet* package (Meyer et al., 2008) for the co-expression estimation with ARACNE. The obtained adjacency matrix was then used to reconstruct a co-expression network for the miRNA targets of *M. truncatula*, with a custom Python (v.2.7) script, exploiting the *NetworkX* package (Hagberg et al., 2008) to create networks in a Cytoscape-compatible format. The

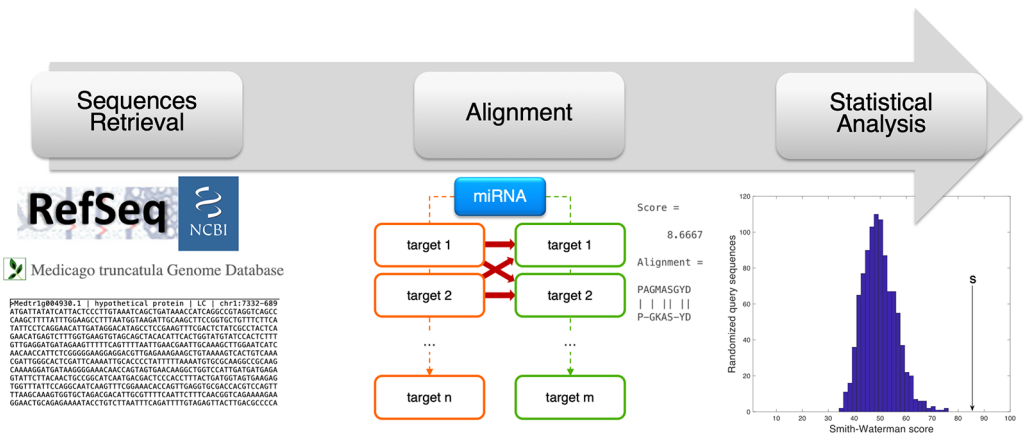

FIGURE 3 | Schematic representation of the alignment-based pipeline. The coding sequence (CDS) and amino acid sequence corresponding to the miRNA target genes were retrieved from online resources (RefSeq and *Medicago truncatula* Genome Database). For each miRNA, the CDSs and amino acid sequences of human and plant targets were compared *via* sequence alignment (Smith-Waterman method, by the swalign Matlab function), to compute a similarity score (provided as swalign output) for each human-plant target pair. The statistical significance of the similarity score is finally computed following a randomization method in which, for every alignment, human sequences (CDS or protein) were randomized and the distribution of swalign scores was used to compute the *p*-value.

miRNA targets of *M. truncatula*, obtained as described above (see *miRNA Target Prediction* section), were mapped onto the network to extract their co-expression interactome. Target genes were mapped and all the co-expressed nodes which interact with at least one miRNA target node were included. The resulting sub-network was filtered, eliminating the smallest components, composed of single nodes or less than ten nodes because these are not informative in terms of interactions. The remaining giant component was considered as the final miRNA target gene network. The giant component was analyzed *via* the gLay clustering procedure and, as performed above for the other networks, the obtained clusters were subjected to the ClueGO enrichment step.

RESULTS

Target Prediction

Following the *in silico* target prediction, a list of 3,468 *M. truncatula* transcripts (2,680 unique transcripts and 2,083 unique genes) was obtained. Conversely, 2,297 target transcripts of *M. truncatula*, corresponding to 1,739 unique transcripts and 1,376 unique genes, were considered for the alignment. Analogously, for the network-based analysis, a list of 936 target transcripts (825 unique transcripts and 758 unique genes) was obtained for *H. sapiens*. For the alignment-based pipeline, 2,226 target transcripts, which correspond to 1,754 unique transcripts and 1,549 unique genes, were obtained. The number of targets was tuned to obtain a balanced number of elements between the two species (see below). The target genes could be associated with one or more than one miRNA, as shown in **Supplementary Figure 1**.

Mtr-miRNAs Targeting Shared Functions Between Plants and Humans From the Perspective of the Network-Based Approach

In this pipeline, we focused on the biological processes enriched among the genes targeted by the set of *M. truncatula* miRNAs to uncover shared functions between plant and human. Following the procedure summarized in **Figure 2**, plant and human miRNA target networks were constructed using the GeneMania web tool. While the construction of the human network was straightforward with this tool, the construction of the plant network relied on the mapping from *M. truncatula* targets to homologous genes in *A. thaliana*, thereby enabling to exploit the deep knowledge (datasets and resources) of *A. thaliana*, since *M. truncatula* is not currently supported by GeneMania. The resulting networks were analyzed using two different topology-based graph clustering methods, to decompose the target network, based on highly connected nodes, implying densely-interacting functional modules. The use of two different clustering methods was devised to increase the sensitivity of the pipeline for the detection of functional modules and, subsequently, associated biological processes. The features of the constructed target gene networks are summarized below:

(1) *A. thaliana*—704 nodes (of which 20 were included by GeneMania as interactors), 13,752 edges, 4 gLay clusters, 6 ClusterOne clusters.
(2) *H. sapiens*—753 nodes (of which 21 were included by GeneMania as interactors), 20,795 edges, 5 gLay clusters, 3 ClusterOne clusters.

The target genes and network clusters obtained in this analysis are reported for each species in the **Supplementary Dataset 1** file.

By performing an enrichment analysis for each cluster, we identified the common biological processes (GO terms) targeted by *M. truncatula* miRNAs in both species. The identified shared biological functions include 'vesicle docking involved in exocytosis' (GO:0006904), 'modulation by virus of host morphology or physiology' (GO:0019048), 'cellular response to virus' (GO:0098586), 'positive regulation of posttranscriptional gene silencing' (GO:0060148), and 'branched-chain amino acid metabolic process' (GO:0009081). The miRNAs and predicted target genes associated with the shared GO terms are listed in **Table 1**. Aside from the identical GO terminologies, other common processes were present in both networks (e.g. nucleic acid and amino acid metabolism, response to stress, signaling) (**Supplementary Dataset 1**).

Exocytosis generally implies the active (hence, energy-dependent) transport of newly synthesized lipids and proteins to the plasma membrane along with the secretion of vesicle-enclosed contents to the extracellular matrix. Experimental evidence that exocytosis-related events can be conserved between plants and animals has been recently provided by Zhang et al. (2016b), who demonstrated that specific molecules (namely endosidin 2) are able to inhibit EXO70 proteins, involved in intracellular vesicle trafficking, in both plants and animals. The fact that *M. truncatula* miRNAs are predicted to target functions related to exocytosis in both *A. thaliana* and *H. sapiens*, further indicate the conservation of these pathways between distant taxa. As an example, the KEU (KEULE) and SEC (EXOCYST COMPLEX COMPONENT) genes in Arabidopsis as well as the human SNPH (Syntaphilin) gene are part of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, which is required for vesicle docking and fusion (Lao et al., 2000; Karnik et al., 2015).

The hypothesis that innate immunity is an evolutionarily conserved process, started in the ancient unicellular eukaryote that pre-dated the divergence of the plant and animal kingdoms (Ausubel, 2005), may explain the shared plant and human response to virus. The network-based approach applied in our study allowed to find common players involved in the response to viral attacks in plants (AGO1, AGO2, DCP2, SDE3, DRD1) and humans (BCL2L11, KPNA4, PUM2, FXR1, RIOK3) (**Table 1**). Particularly, the KPNA4 (Karyopherin Subunit Alpha 4) mediates the nuclear import of human cytomegalovirus UL84 (Lischka et al., 2003), PUM2

TABLE 1 | Common biological processes shared between *A. thaliana* and *H. sapiens* as resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	GO ID	*A. thaliana*		*H. sapiens*	
		Gene	miRNA	Gene	miRNA
Vesicle docking involved in exocytosis	GO:0006904	EXO70B1	mtr-miR5244	SNPH	mtr-miR399t-5p
		EXO70D1	mtr-miR2653a		
		EXO70H7	mtr-miR397-5p		
		KEU	mtr-miR5559-3p		
		SEC5A	mtr-miR7698-5p		
		SEC8	mtr-miR2679a		
Modulation by virus of host morphology or physiology	GO:0019048	AGO2	mtr-miR2673a	BCL2L11	mtr-miR5273
		DCP2	mtr-miR5238	KPNA4	mtr-miR169k
			mtr-miR2655b		
Cellular response to virus	GO:0098586	AGO1	mtr-miR168a	BCL2L11	mtr-miR5273
		SDE3	mtr-miR168c-5p	PUM2	mtr-miR160c
			mtr-miR2592a-3p	RIOK3	mtr-miR160a
			mtr-miR2592bm-3p		
Positive regulation of posttranscriptional gene silencing	GO:0060148	DRD1	mtr-miR2650	FXR1	mtr-miR482-3p
				PUM2	mtr-miR160c
Branched-chain amino acid metabolic process	GO:0009081	BCAT3	mtr-miR5212-3p	BCKDK	mtr-miR5273
		CSR1	mtr-miR2660	IVD	mtr-miR2640

(Pumilio RNA Binding Family Member 2) plays a role in cytoplasmic sensing of viral infection (Narita et al., 2014), and RIOK3 (right open reading frame-RIO Kinase 3) is involved in regulation of type I interferon (IFN)-dependent immune response, with a critical role in the innate immune response against DNA and RNA viruses (Feng et al., 2014).

The relationship between miR168a and AGO1 (ARGONAUTE) has been long studied and experimentally validated in plants (Vaucheret et al., 2006), whereas several other targets of the plant miR168a have been identified and/or validated in humans (Zhang et al., 2012; Javed et al., 2017). Aside being involved in miRNA biogenesis and regulation (Mallory and Vaucheret, 2010), AGO proteins have a myriad of other functions including plant antiviral responses and DNA repair (AGO2) (Harvey et al., 2011; Oliver et al., 2014; Carbonell and Carrington, 2015), miRNA-directed target cleavage (AGO5), and RNA-directed DNA methylation (AGO9) (Oliver et al., 2014). Differently, the validated osa-miR168a target in humans is LDLRAP1, with functions in cholesterol metabolism (Zhang et al., 2012), while other predicted targets included RPL34 (Large Ribosomal Subunit Protein EL34), ATXN1 (Ataxin-1), and ALS2 (Alsin Rho Guanine Nucleotide Exchange Factor) with roles in transcription, ribosome biogenesis, and cell trafficking (Javed et al., 2017). Other genes (ST8SIA1, RGS6, IL18RAP, PVR, SYN2, PPFIA1, ZDHHC18, B3GAT1) were predicted in our network-based pipeline to be targeted by mtr-miR168 in humans (**Supplementary Table 1**). This may be due to the fact that, even if miR168a is part of conserved miRNA family, some differences in nucleotide sequences are present among monocot and dicot species and these can alter the structural accessibility and target selection (Lang et al., 2019). When aligning the osa-miR168a with its counterpart in *M. truncatula*, the sequence similarity was of 80.95%, showing important mismatches in the seed region (**Supplementary Figure 2**). This, along with the fact that we took into consideration only the 3′ UTR region, explains the absence of LDLRAP1 among the mtr-miR168a targets in human. We confirmed this by comparing the *M. truncatula* (mtr-) or rice (osa-) miR168a targets found within the human transcript collection (retrieved from NCBI RefSeq) or in the 3′ UTRome, as performed in our pipeline. As expected, we found that no target was detected for both miRNAs in the 3′ UTRome, while targets in the LDLRAP1 coding sequence were found with relevant MFE. In particular, osa-miR168a showed a −35.3 kcal/mol MFE with LDLRAP1, which appeared in the top 5 of the miRNA targets, while mtr-miR168a showed a −33.8 kcal/mol MFE with LDLRAP1 in the 100th position of the lowest-MFE target list (**Supplementary Figure 2**).

miRNAs Targeting Shared Functions in *M. truncatula* and *H. sapiens* Through the Lens of the Alignment-Based Approach

Unlike the network-based pipeline in which over-represented biological processes were searched in the network of all the miRNA target genes, here we focus on sequence similarities among the targets of a given miRNA. In this approach, the analysis included alignments of every single targeted gene (and corresponding protein sequence) between *M. truncatula* and *H. sapiens*, resulting in a total number of 9,626 alignments (**Supplementary Dataset 2**). By applying a threshold *p*-value of 0.05 for nucleotide alignments, 2,735 sequences, corresponding to 115 miRNAs, resulted significant. These miRNAs were predicted to target a total of 315 genes in *M. truncatula* and 801 genes in *H. sapiens*, respectively. Similarly, when this threshold was applied

for the protein alignments, from 697 alignments (including 81 miRNAs) 352 genes were identified in *H. sapiens* and 192 in *M. truncatula*. When considering both the gene and protein sequences, 242 similarities between plant and human transcripts were found, accounting for 93 genes (targeted by 54 miRNAs) in *M. truncatula* and 149 in *H. sapiens* (**Supplementary Dataset 2**).

Focusing on the identification of genes involved in similar functions between the two organisms, the main hits were related to transcription factors (including zinc finger proteins), hormone-responsive elements, and cell division (**Table 2**). The activity of transcription factors (TFs), consisting of the interaction with enhancers to coordinate gene expression, is a common denominator for all living forms. In eukaryotes, another level of regulation is given by miRNAs; these are known to target mostly TFs, at least in plants (Samad et al., 2017). Moreover, coordinated action of TFs and phytohormones guide most plant developmental processes as well as cellular proliferation and dedifferentiation (Long and Benfey, 2006). The predicted targets of miR164 belong to CUP and NAC families of TFs, and these had been previously validated in plants in other works (Fang et al., 2014). However, a piece of interesting information is the fact that this miRNA could target TFs also in human cells. For instance, ZXDC (predicted as a target of mtr-miR164b), belonging to the zinc finger X-linked duplicated (ZXD) family of TFs, is involved in the regulation of histocompatibility (Ramsey and Fontes, 2013). Elseway, HAND2 (Heart and Neural Crest Derivatives Expressed 2), putatively targeted by mtr-miR2673a, is a member of the helix-loop-helix family of TFs involved in cardiac morphogenesis, vascular development, and regulation of angiogenesis (McFadden et al., 2005). Another interesting fact is that this analysis predicted that conserved miRNA families (miR160, miR166) target genes with roles in hormone regulation in both *M. truncatula* (ABA response element-binding factor, auxin response factor) and *H. sapiens* (DYRK1B, HNF4A). In humans, DYRK1B (Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1B), encoding for a nuclear-localized protein kinase, and HNF4A (Hepatocyte Nuclear Factor 4 Alpha), belonging to the nuclear hormone receptor family, are associated with steroid hormone activity (Sladek et al., 1990; Sitz et al., 2004). Other interesting hits revealed through this approach are presented in **Supplementary Table 2**. An example is represented by mtr-miR2600e, predicted to target an anthocyanin acyltransferase (Medtr2g089765) in *M. truncatula* and the UVSSA (UV Stimulated Scaffold Protein A) gene in *H. sapiens*. Anthocyanins are well-known secondary metabolites with antioxidant function, being able to mitigate photooxidative injury (e.g. UV irradiation) at the cellular and nuclear level by efficiently scavenging reactive oxygen species (Gould, 2004). UVSSA encodes a protein involved in ubiquitination and dephosphorylation of RNA polymerase II subunits, being involved in the transcription-coupled nucleotide excision repair (TC-NER) pathway associated with UV irradiation (Schwertman et al., 2013).

When comparing the network-based and alignment-based approaches, in the case of mtr-miR168a targets, it is possible to evidence the same predicted target in plants (AGO1) along with different predicted targets in humans (**Supplementary Table 2**, **Supplementary Dataset 2**). However, drawing the attention to the 'response to virus' function, it is possible to observe that this was hit with both approaches, as demonstrated by the common predicted target gene PVR (Poliovirus Receptor).

Novel Co-Expression Network Reveals Shared Functions Targeted by mtr-miRNAs in Both *M. truncatula* and Humans

The third approach used in this study pursued the construction of a new *M. truncatula* co-expression network using publicly available gene expression microarray datasets since this organism is not currently supported in readily usable bioinformatic tools for network analysis and construction. An expression panel of 24,777 genes was obtained and used to build a genome-scale co-expression network for *M. truncatula*. The resulting 24,777-node network had 62,857 undirected edges (**Figure 4A**). Among the 2,083 predicted target genes, 1,251 were mapped in this network, resulting in a sub-network of 6,081 nodes and 9,534 edges. The giant component of this sub-network included 5,943 nodes (of which 1,208 were target genes) and 9,405 edges (of which 3,102 were direct interactions among miRNA target nodes), as shown in **Figure 4B**. The clustering procedure found

TABLE 2 | Mtr-miRNAs and their putative target genes related to similar functions in *M. truncatula* and *H. sapiens* as revealed by the alignment-based approach. The genes and their respective accessions are provided for each organism.

mtr-miRNA	*M. truncatula*		*H. sapiens*	
	Accession	Gene	Accession	Gene
mtr-miR166d	Medtr2g086390	ABA response element-binding factor	NM_006484	DYRK1B
mtr-miR160a	Medtr5g061220	auxin response factor	NM_175914	HNF4A
mtr-miR2673a	Medtr2g014260	zinc finger C-x8-C-x5-C-x3-H type protein	NM_001170538	DZIP1L
mtr-miR2673a	Medtr4g082580	WRKY transcription factor 3	NM_021973	HAND2
			NM_032772	ZFN503
mtr-miR164b	Medtr2g078700	CUP-shaped cotyledon protein, putative	NM_001099694	ZNF578
	Medtr4g108760		NM_001040653	ZXDC
mtr-miR164d	Medtr3g435150	NAC transcription factor-like protein	NM_001018052	POLR3H
mtr-miR5287b	Medtr7g088980	cell division cycle protein-like/CDC48 protein	NM_001277742	CYP26B1

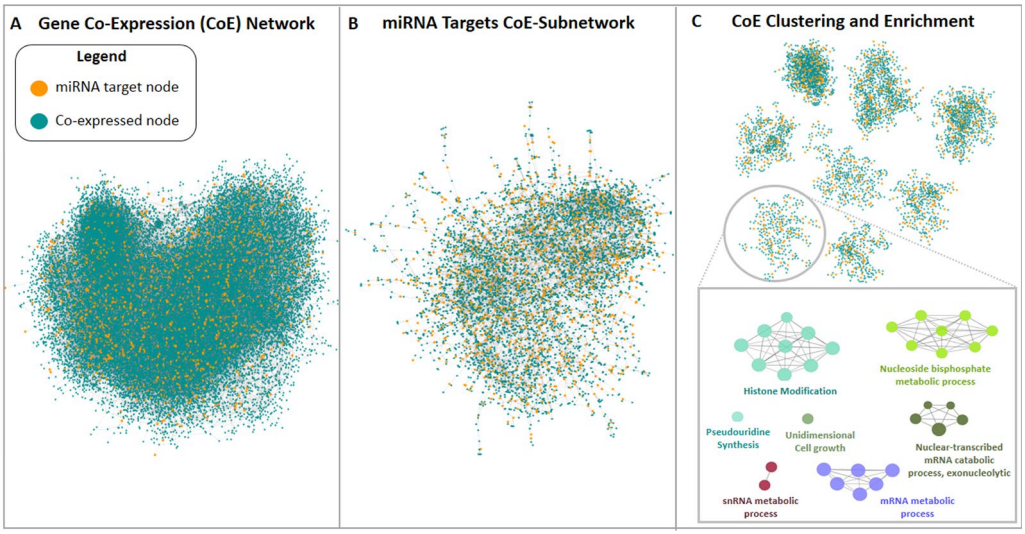

FIGURE 4 | *Medicago truncatula* co-expression network construction and analysis pipeline. **(A)** Genome-scale co-expression network and **(B)** miRNA targets network are shown, where blue nodes represent genes not found among miRNA targets, while orange nodes are miRNA targets. **(C)** Representative set of the clusters resulted from the miRNA targets network analysis. Each cluster was analyzed *via* enrichment analysis using ClueGO.

45 clusters (**Figure 4C**) which were analysed *via* enrichment analysis. All the resulting GO terms along with the co-expressed genes and associated miRNAs are reported in the **Supplementary Dataset 3** file.

The herein generated network was compared with the first network-based approach made with the tools available for *A. thaliana* to evaluate if the two different network construction procedures lead to the same target biological processes, thereby assessing the robustness of the conclusions for the network-based approach. The GO terms identified within the *M. truncatula* network were mainly related to general processes such as metabolic pathways (e.g., nucleic acids, proteins, and carbohydrates metabolism), plant development (e.g., fruit, seed, embryo development), or hormone signaling (**Supplementary Dataset 3**). On the other hand, the Arabidopsis network was much more varied and specific (**Supplementary Dataset 1**), mostly because *A. thaliana* is *de facto* considered the plant model *par excellence* and hence, much more information, databases, and bioinformatics resources are available in this case. Despite a systematic comparison between the biological processes in the two plants could not be carried out, identical GO terms identified between *A. thaliana* and *M. truncatula* include exocytosis, folic acid metabolism, and thylakoid membrane organization (**Supplementary Table 3**). In this case, it can be underlined also the fact that some miRNAs (e.g., mtr-miR5559-3p, mtr-miR5558-3p, mtr-miR2662, mtr-miR5212-3p) are predicted to target the same genes/functions in both plant species. When comparing the shared biological processes between *M. truncatula* and *H. sapiens*, these are shown to be related to exocytosis, DNA replication, transcription, and modifications, amino acid activation and transport, RNA related processes, histone modification, and protein modifications (**Table 3**). To cite one example, histone modification functions associated with both organisms include the DNA methyltransferase 1-associated protein (Medtr1g086590) in plants and the KANSL1 (KAT8 Regulatory NSL Complex Subunit 1) histone acetyltransferase in humans.

Taken together, the results obtained confirmed that both network-based approaches lead to consistent conclusions, even if *M. truncatula* is characterized by a less detailed Gene Ontology which prevents strong matching between the two plants.

Do mtr-miRNAs Putatively Target Genes Involved in DDR in Plants and Humans?

The three bioinformatic approaches used in the present study allowed to search for common biological processes targeted by *M. truncatula* miRNAs in both plant and human cells. Each approach provided different sets of information that can be either complementary or divergent, based on the assumptions of each used methodology. Besides the results presented so far, we also wanted to focus on a particularly conserved pathway in plants and humans, namely DDR (Yoshiyama et al., 2013; Nikitaki et al., 2018), because information relative to miRNAs targeting this essential process is still scarce, especially when concerning plants. Hence, **Tables 4** and **5** summarize a series of processes related to the DDR pathway and downstream processes in both kingdoms.

DNA repair, recombination, replication, and chromatin dynamics are tightly connected, as modifications of DNA conformation is required in order to allow access of the repair machinery to the damaged sites. This interplay is evidenced also by the fact that several genes are shared among these processes; for instance, the *A. thaliana* DME (Demeter) and DML1 (Demeter-like 1) are associated with both DNA repair (BER-base excision repair, GO:0006284) and chromatin

TABLE 3 | Common biological processes shared between *M. truncatula* and *H. sapiens* as resulted from the network-based approach involving the *M. truncatula* network construction. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*M. truncatula*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
Exocytosis	GO:0006887	Medtr4g102120	mtr-miR5559-3p	GO:0006887	SNPH	mtr-miR399t-5p
		Medtr8g023330	mtr-miR5558-3p	GO:0006904	RIMS3	mtr-miR482-5p
					SYT1	mtr-miR5211
					SYT2	mtr-miR2640
					NOTCH1	mtr-miR5266
					RAB3GAP1	mtr-miR5209
					RPH3AL	mtr-miR2589
					SYT15	mtr-miR166d
DNA replication, transcription, and modifications	GO:0006261	Medtr4g106540	mtr-miR5741a	GO:0090329	INO80	mtr-miR399t-5p
	GO:0090329			GO:2000104	LIG3	mtr-miR5294a
				GO:0006268	HMGA1	mtr-miR5276 mtr-
				GO:0044030	GRHL2	miR2589
				GO:2000678	PER2	mtr-miR169k
				GO:0032786	SIN3A	mtr-miR156b-3p
					BRD4	mtr-miR5266
Amino acid activation and transport	GO:0043038	Medtr7g083030	mtr-miR2657	GO:0009081	BCKDK	mtr-miR5273
	GO:0043039			GO:0009083	IVD	mtr-miR2640
	GO:0006418			GO:0051955	PER2	mtr-miR169k
				GO:0051957	RAB3GAP1	mtr-miR5209
				GO:0009065	NTSR1	mtr-miR408-3p
					TINAGL1	mtr-miR166d
					NANOS2	mtr-miR160c
					PRODH	mtr-miR169d-3p
RNA related processes	GO:0016071	Medtr3g077320	mtr-miR2629f	GO:0050686	CELF1	mtr-miR2670f
	GO:0006397			GO:0006376	CELF2	mtr-miR399t-5p
	GO:0008380			GO:0061014	GIGYF2	mtr-miR166d
	GO:0000375			GO:0061157	TNRC6B	mtr-miR5211
	GO:0000377			GO:0050686	KHSRP	mtr-miR398b
	GO:0000398				MEX3D	mtr-miR2673a
					RNPS1	mtr-miR398b
					SUPT5H	
Histone modification	GO:0016570	Medtr1g086590	mtr-miR395e	GO:0043981	KANSL1	mtr-miR482-5p
	GO:0016573	Medtr4g108080	mtr-miR156a	GO:0043982		
Protein modifications	GO:0043543	Medtr1g086590	mtr-miR395e	GO:0018345	CLIP3	mtr-miR527
	GO:0006473			GO:0006517	ZDHHC18	mtr-miR168b
	GO:0006475			GO:0036507	MARCH6	mtr-miR390
	GO:0018394			GO:0036508	UGGT1	mtr-miR5270a
	GO:0018393			GO:0042532	NF2	mtr-miR5206b

modification-related functions (GO:0006306, GO:0044728), whereas RAD54 (DNA Repair and Recombination Protein), RECA1 (Recombination A1 protein), and KU80 (helicase Ku80 subunit of KU complex) are coupled with DNA repair (DSB, double-strand break repair, GO:0045003; HR-homologous recombination, GO:0000724) and DNA recombination (GO:0006310) processes (**Table 4**). Similarly, literature available from medical research assigned roles in DNA damage repair and chromatin remodeling to some of the genes predicted as targets of mtr-miRNAs. To cite some examples, PPP4C (Protein Phosphatase 4 Catalytic Subunit), is involved in a myriad of processes spanning from microtubule organization, to apoptosis, DNA repair, DNA damage checkpoint signaling, regulation of histone acetylation (Zhou et al., 2002; Lee et al., 2010), while INO80 (INO80 Complex Subunit) is the catalytic ATPase subunit of the INO80 chromatin remodeling complex, being however related also to DNA DSB repair (Conaway and Conaway, 2009). Functions related to DNA and chromatin/histone modifications were identified also in the *M. truncatula* network-based approach (see **Table 3**). This is the case of Medtr1g086590 (DNA methyltransferase 1-associated protein), Medtr4g108080 (ubiquitin-conjugating enzyme), and Medtr4g106540 (E2F transcription factor-E2FE-like protein) accessions. Within the alignment-based approach, mtr-miR2589 was predicted to target the *M. truncatula* Medtr6g047800 (tRNA methyltransferase complex GCD14 subunit) and the *H. sapiens* SETD1A (SET Domain Containing 1A, Histone Lysine Methyltransferase), functions involved in chromatin organization in both organisms (**Supplementary Table 2**).

Other processes tightly correlated with DDR include cell cycle and cell death (apoptosis/necrosis/programmed

TABLE 4 | Biological processes related to DNA repair, recombination, replication and chromatin remodeling common to *A. thaliana* and *H. sapiens* as resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*A. thaliana*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
DNA repair	GO:0006284	DME	mtr-miR2086-3p	GO:2000779	FOXM1	mtr-miR169d-3p
	GO:0045003	DML1	mtr-miR2651		PPP4C	mtr-miR169k
	GO:0000724	AT1G75230	mtr-miR5240			
		RAD54	mtr-miR172c-5p			
		RECA1	mtr-miR5558-3p			
		ASF1B	mtr-miR1509a-3			
		GMI1	pmtr-miR169l-3p			
		KU80	mtr-miR5272f			
DNA recombination and replication	GO:0006310	ASF1B	mtr-miR1509a-3p	GO:0090329	INO80	mtr-miR399t-5p
		GMI1	mtr-miR169l-3p	GO:2000104	LIG3	mtr-miR5294a
		KU80	mtr-miR5272f	GO:2000678	PER2	mtr-miR169k
		RAD54	mtr-miR172c-5p	GO:0032786	SIN3A	mtr-miR156b-3p
		RCK	mtr-miR5754		BRD4	mtr-miR5266
		RECA1	mtr-miR5558-3p			
		RPA70B	mtr-miR2592a-5p			
Chromatin remodeling	GO:0006306	DME	mtr-miR2086-3p	GO:0043981	KANSL1	mtr-miR482-5p
	GO:0044728	DML1	mtr-miR2651	GO:0043982	HMGA1	mtr-miR5276
	GO:0006305	DRD1	mtr-miR2650	GO:0070828	TNRC18	mtr-miR2589
	GO:0006304	EMB2770	mtr-miR7696c-5p	GO:0031507	GRHL2	mtr-miR2589
	GO:0031056	SDG14	mtr-miR2650	GO:0031936	PHF2	mtr-miR160c
	GO:0031058		mtr-miR2086-3p	GO:0006268	SIN3A	mtr-miR156b-3p
	GO:0031060		mtr-miR7696c-5p	GO:0044030	ZNF304	mtr-miR166e-5p
	GO:0031062			GO:0031935		
	GO:1905269			GO:0031937		
	GO:1902275					
	GO:0080188					

cell death). DNA replication, recombination, and repair are more active during certain phases of the cell cycle and the success of these processes can decide the fate of the cell. The connection between pathways is evidenced by genes that play important functions in both DNA repair/replication, chromatin remodeling, and cell cycle/cell death (**Table 5**). This is the case of the ASF1B (Anti-Silencing Function 1B, histone chaperone) and KU80 functions in plants, involved in the S-phase replication-dependent chromatin assembly (Zhu et al., 2011) and maintenance of genome integrity (West et al., 2002), respectively, and the SIN3A (Histone Deacetylase Complex Subunit Sin3a) and HMGA1 (High Mobility Group Protein A1) genes in humans, with roles associated to chromatin regulation and cell cycle progression (Silverstein and Ekwall, 2004; Pierantoni et al., 2015). While the *A. thaliana* network-based approach has not identified genes (predicted targets of mtr-miRNAs) associated with cell death in plants, this however led to the identification of many putative targets related to apoptosis in human cells. To cite a few, BCL2L11 (BCL2-Like 11 apoptosis facilitator), NOTCH1 (Notch Receptor 1), and TP53BP2 (Tumor Protein P53 Binding Protein 2) are among the most essential apoptotic factors. NOTCH1, part of the Notch signaling pathway, is involved in many processes related to cell fate specification, differentiation, proliferation, and survival, while its activation is related to many types of cancers (e.g. cervical, colon, head and neck, lung, renal, pancreatic, leukemia, and breast cancer) (Xiao et al., 2016). Hence, dietary miRNAs targeting this specific function may have positive implications in sustaining cancer therapies. The alignment-based approach allowed to identify a conserved miRNA (mtr-miR319d-5p) predicted to target genes associated with cell death functions in both *M. truncatula* (DCD-development and cell death domain protein) and *H. sapiens* (MESD, PRR5L) (**Supplementary Table 2**). While MESD (Mesoderm Development LRP Chaperone) is related to the Notch pathway (Hsieh et al., 2003), PRR5L (Proline Rich 5 Like) regulates the activity of the mTORC2 (mechanistic target of rapamycin) complex controlling cell migration (Gan et al., 2012).

Hence, to answer the herein proposed question, the network-based as well as the alignment-based approaches pinpointed mtr-miRNAs predicted to target genes involved in DDR and downstream processes in *A. thaliana*, *M. truncatula*, and *H. sapiens*.

DISCUSSION

In view of the controversies raised by the recent 'dietary xenomiR' hypothesis (Witwer, 2012), bioinformatics studies have the potential to aid the ongoing efforts to reinforce new methodologies and provide the basis for further experimental validation. Model organisms, like *A. thaliana*, are used as guidance systems to explore bioinformatics data-driven questions related

TABLE 5 | Biological processes related to cell cycle and cell death common to *A. thaliana* and *H. sapiens* as resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*A. thaliana*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
Cell cycle	GO:0000075	ASF1B	mtr-miR1509a-3p	GO:1901989	BRD4	mtr-miR5266
	GO:0045930	RAD9	mtr-miR2638b	GO:1901992	EIF4G1	mtr-miR166d
	GO:0007093			GO:1902751	PHB2	mtr-miR5266
				GO:0010971	SIN3A	mtr-miR156b-3p
				GO:0071157	MDM2	mtr-miR169k
					MDM4	mtr-miR5266
Cellular senescence	GO:0000723	KU80	mtr-miR5272f	GO:2000772	ABL1	mtr-miR5276
		TRB1	mtr-miR5558-5p		HMGA1	mtr-miR5276
					VASH1	mtr-miR160c
Apoptosis/cell death	–	–	–	GO:1902108	BMF	mtr-miR2613
				GO:1902110		mtr-miR5266
				GO:1902263	GDNF	mtr-miR2673a
				GO:0060561	BCL2L11	mtr-miR5273
				GO:0001844		mtr-miR5266
				GO:1900117	NOTCH1	mtr-miR5266
				GO:0070231	VDR	mtr-miR5276
				GO:0043525	YWHAG	mtr-miR2673a
				GO:1901028		mtr-miR399t-5p
				GO:1901216	DFFA	mtr-miR399t-5p
				GO:1901030	TP53BP2	mtr-miR399t-5p
				GO:2001238		mtr-miR5266
				GO:0097192		mtr-miR2613
				GO:1900740		mtr-miR160c
				GO:1902686	AKT1	mtr-miR160c
						mtr-miR5266
					DFFA	mtr-miR399t-5p
					KDELR1	mtr-miR166d
					ARHGEF7	mtr-miR2589
					GDNF	mtr-miR2673a
					BAD	mtr-miR5266
						mtr-miR5276
						mtr-miR399t-5p
						mtr-miR2673a
					ABL1	mtr-miR399t-5p
					ITM2C	mtr-miR5206a
					PEA15	mtr-miR160c
					TNFRSF12A	mtr-miR160c
					TRAF2	mtr-miR2673a
					CX3CL1	mtr-miR2613
					GDNF	mtr-miR5211
					SPG7	mtr-miR399t-5p

to putative cross-species miRNA targets (Zhang et al., 2016a). The high interest in this field prompted the development of databases able to predict the functional impact of food-borne miRNAs in humans (Chiang et al., 2015; Shu et al., 2015). The DMD (Dietary MicroRNA Database) database covers only very few edible plant species (Chiang et al., 2015), hence there is the need to substantially enlarge the information and include alternative species with a potential impact on food security. Within this framework, the current study aims at identifying plant miRNAs along with their endogenous and cross-kingdom targets to pinpoint conserved pathways between evolutionary distant species. Starting from a list of publicly available *M. truncatula* miRNAs, we made the assumption that any miRNA may have the potential to target genes in both plants and humans. Given that the bioinformatics approaches do not allow the prediction of miRNAs stability and function validation within the organisms, there is the need to further experimentally confirm the proposed hypotheses. The choice of plant species is driven by the fact that *M. truncatula* is at the crossroad between model organisms (in the case of legume research) and economically relevant species, given its potential use as microgreens to support more sustainable agriculture. The presented methodologies can serve both as guidelines to be applied to other plant species as well as to test new hypotheses exploring the potential benefits of food-borne mtr-miRNAs targeting human genes. When considering the conserved families of miRNAs, this study could aid the translational research covering other economically relevant plant species (with 100% sequence similarity) and potential human target genes. As exemplified in **Figure 5**, miR164, miR166, and miR390 have a 100% sequence similarity between *M. truncatula* and other dicot plant species such as tomato (*Solanum lycopersicum*) and apple (*Malus domestica*). Among the selected

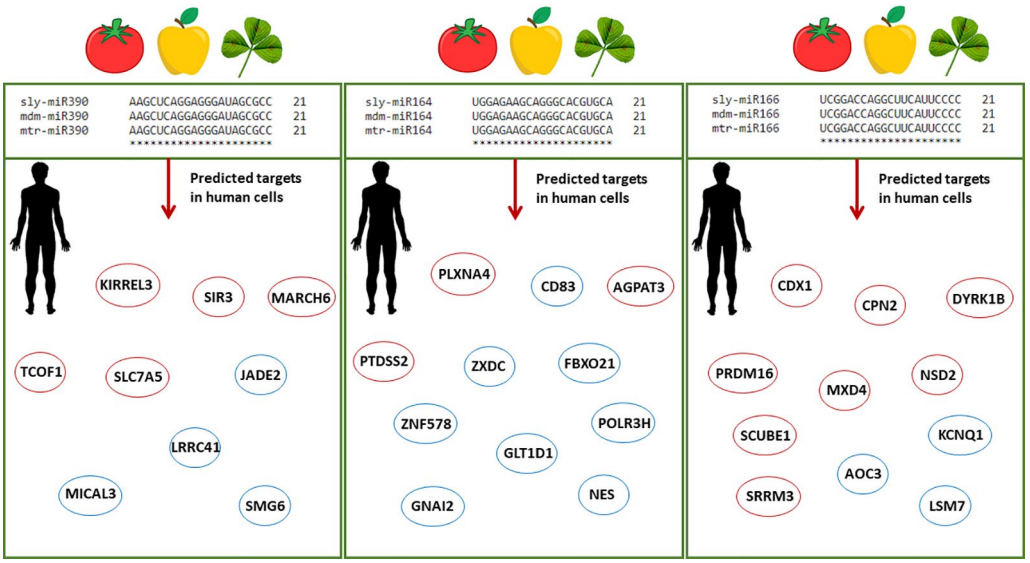

FIGURE 5 | Schematic representation of conserved plant miRNAs potentially targeting human genes. Alignments between three conserved miRNAs (miR390, miR164, miR166) from different plant species, namely *Solanum lycopersicum* (sly), *Malus domestica* (mdm), and *M. truncatula* (mtr), show 100% sequence similarity. The predicted human target genes found in the enriched biological processes of the network-based approach and among the genes with significant sequence similarity in the alignment-based approach are shown in red and blue circles, respectively. Abbreviations: KIRREL3, Kirre Like Nephrin Family Adhesion Molecule 3; SIRT3, Sirtuin 3; MARCH6, Membrane Associated Ring-CH-Type Finger 6; TCOF1, Treacle Ribosome Biogenesis Factor 1; SLC7A5, Solute Carrier Family 7 (Amino Acid Transporter Light Chain, L System), Member 5; JADE2, Jade Family PHD Finger 2; LRRC41, Leucine Rich Repeat Containing 41; MICAL3, Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 3; SMG6, Nonsense Mediated MRNA Decay Factor; PLXNA4, Plexin A4; AGPAT5, 1-Acylglycerol-3-Phosphate O-Acyltransferase 5; PTDSS2, Phosphatidylserine Synthase 2; CD83, Cluster of Differentiation 83; ZXDC, ZXD Family Zinc Finger C; FBXO21, F-Box Protein 21; GLT1D1, Glycosyltransferase 1 Domain Containing 1; GNAI2, G Protein Subunit Alpha I2; ZNF578, Zinc Finger Protein 578; NES, Nestin; POLR3H, RNA Polymerase III Subunit H; CDX1, Caudal Type Homeobox 1; CPN2, Carboxypeptidase N Subunit 2; DIRK1B, Dual specificity tyrosine-phosphorylation-regulated kinase 1B; MXD4, MAX Dimerization Protein 4; NSD2, Nuclear Receptor Binding SET Domain Protein 2; PRDM16, Histone-lysine N-methyltransferase PR/SET Domain 16; SCUBE1, Signal Peptide, CUB Domain And EGF Like Domain Containing 1; SRRM3, Serine/Arginine Repetitive Matrix 3; AOC3, Amine Oxidase Copper Containing 3; KCNQ1, Potassium Voltage-Gated Channel Subfamily Q Member 1; LSM7, U6 snRNA-associated Sm-like protein 7.

examples, miR166 was previously demonstrated to be abundant in different human body fluids and tissues (Lukasik et al., 2018; Zhao et al., 2018b). The putative human targets identified either through the network- or alignment-based approaches could serve as potential candidates to aid medical interventions. To cite one example, inhibition of the AOC3 (Amine Oxidase Copper Containing 3), playing important roles in adipogenesis and putatively targeted by miR166, could result in decreased fat deposition (Carpéné et al., 2007; Shen et al., 2012), hence addressing the big issues related to obesity and the many obesity-associated diseases.

It is important to underline that the experimental design of this study was thought in such a way to potentiate the identification of conserved pathways/players between evolutionary distant species. To do so, three different bioinformatics pipelines were developed (two network-based approaches, considering *A. thaliana* and *M. truncatula* model species, and one alignment-based approach) to confront plant and human targeted biological processes. Form a methodological point of view, the developed approaches enable the exploration of different assumptions supported by robust statistical methods. The network-based approaches rely on extensive knowledge available on the interactions among miRNA target genes in a given species. The knowledge was exploited for the construction of gene co-expression/interaction networks, from which a set of biological processes predicted to be targeted by the plant miRNAs were found. This approach aims to study the regulatory potential of the full list of *M. truncatula* miRNAs. Two network-based approaches were implemented, differing from the point of view of plant network construction. In one approach, the predicted *M. truncatula* target genes were mapped to the genome of *A. thaliana*, which is a supported organism in widely used network construction tools, while *M. truncatula* is not. The other network-based approach relies on the construction of a novel co-expression network for *M. truncatula*, using publicly available expression data, thereby evaluating the robustness of the performed assumptions on the plant network. Finally, the alignment-based approach was radically different, since it only relies on target gene and protein sequences, with no other assumption, and aimed to discover sequence similarities between plant and human targets, individually for each targeting miRNA. In this context, this approach enabled the inference on the potential effect of every single miRNA of the initial list. Importantly, none of the proposed strategies is focused on the prediction of individual target genes: only the ones sharing statistically over-represented processes (in network-based approaches), or having the same targeting miRNA and a statistically significant nucleotide and protein sequence similarity (in alignment-based approaches) were detected and discussed in

this work. The prediction of individual targets relied on existing computational tools used in the early steps of the workflow under assumptions similar to the ones of previous studies (Zhang et al., 2016a), despite no standardized pipeline is well-accepted to accomplish this task due to the lack of genome-wide experimental validations across species. The main limitations of the proposed approaches are that: *(i)* no assumption was made on which miRNAs can be delivered between plants and human, *(ii)* only the 3′ UTR region was assumed to be the target region of plant miRNAs in humans. In addition, we decided to rely on computational methods to predict the binding between miRNA and putative target transcript; alternative approaches could also exploit homology between plant and human miRNAs, which might share the same seed region and then identify human targets based on experimentally validated target genes in human cells (Liu et al., 2017). However, such alternative would have led to the consideration of a smaller number of plant miRNAs, since only the ones with homologous features could have been included in the analysis, thereby losing the possibility to study the whole plant miRNA regulation potential. Nonetheless, we believe that the methodological approaches are sufficiently general to be extended onto the desired miRNA list and candidate target list (e.g. 3′/5′ UTRome, transcriptome, or collection of coding sequences) as input. Moreover, the interpretation of the enriched biological processes identified from network analysis is affected by Gene Ontology terms of different specificity and name (Zhang et al., 2016a), thereby limiting the discovery of all the potentially targeted functions.

From a biological perspective, the employed strategies resulted in both complementary and divergent observations. For instance, 'exocytosis' was a common denominator in all three investigated species (*M. truncatula*, *A. thaliana*, and *H. sapiens*) when using the network-based approaches. On the other hand, the alignment-based approach allowed a more direct identification of miRNAs targeting genes in *M. truncatula* and *H. sapiens* (e.g., same miRNA *vs.* similar/different functions) whereas the generated networks illustrates conserved biological processes (e.g., same function *vs.* same/different miRNAs). The two approaches also indicated connecting elements. For example, mtr-miR168a was predicted to target AGO1 in plants and PVP in humans, functions associated with pathogen (namely, viruses) defence, in both approaches. The miR168a is part of a conserved family of plant miRNAs among different species, but as we seen in **Supplementary Figure 2** and other cited literature (Lang et al., 2019), differences exist between dicot and monocot species. The predicted human targets observed in previously published researches (Zhang et al., 2012; Javed et al., 2017), were not found in the enriched process or sequence similarity with our approaches (see **Supplementary Table 1**). This can have different explanations: *(i)* four sequence mismatches (two located within the seed region) are present between osa-miR168a and mtr-miR168a, including a G at position 14, recently reported to generate a G:U wobble that limits its binding to LDLRAP1 (Lang et al., 2019); *(ii)* only 3′ UTR regions were considered in our study, and since osa-miR168a targets the LDLRAP1 CDS region (Zhang et al., 2012), we did not find this match in the target list; *(iii)* we used the entire length of the miRNA and 100% sequence complementarity instead of only the miRNA seed region (Zhang et al., 2012; Javed et al., 2017). By searching for mtr-miR168a and osa-miR168a targets in the full transcript sequences, a more relevant annealing score to the LDLRAP1 gene was found with osa-miR168a than mtr-miR168a, probably due to the sequence mismatches.

Considering that the purpose of the study was to identify conserved functions between distant species through the lens of mtr-miRNAs, our results report 'vesicle docking involved in exocytosis', 'modulation by virus of host morphology or physiology', 'cellular response to virus', 'positive regulation of posttranscriptional gene silencing', and 'branched-chain amino acid metabolic process' as common biological processes between Arabidopsis and humans (**Table 1**). A different study designed to look into the role of plant miRNAs in inter-species regulatory networks indicated ion transport and stress response as shared functions between Arabidopsis and humans (Zhang et al., 2016a). However, this study took into consideration only 25 miRNAs to construct the relative species-specific networks while we started from a list of 426 *M. truncatula* miRNAs to disclose the full regulatory potential. When considering the alignment-based approach, the most represented predictive targets in *M. truncatula* covered transcription factors and hormone-responsive genes. Interestingly, some of these miRNAs (e.g., mtr-miR160a, mtr-miR164b, mtr-miR166d, mtr-miR2673a) were predicted to target TFs (HAND2, ZXDC) and hormone-related functions (DYRK1B, HNF4A) also in human cells. This is in agreement with the concept that miRNAs may behave in a hormone-like manner since hormones and miRNAs are reciprocally regulated in both plant and animal kingdoms (Li et al., 2018).

Because evidence of miRNAs involvement in the regulation of DDR-related pathways is still limited in plants and considering the conservation of some DDR functions between plants and animals (Yoshiyama et al., 2013; Nikitaki et al., 2018), we decided to focus our attention on these specific pathways. Hence, miRNAs predicted to target genes involved in DNA repair, recombination, and replication, chromatin remodeling, cell cycle, and cell death were hereby identified in plants (see **Tables 4** and **5**). For instance, mtr-miR172c-5p, mtr-miR2638b, mtr-miR5272f, and mtr-miR2086-3p, were predicted to target the Arabidopsis RAD54, RAD9, KU80, and DME genes, respectively. In the *M. truncatula* network-based approach, the 'DNA-dependent DNA replication' (GO:0006261) biological process is represented by Medtr4g106540 (E2F transcription factor-E2FE-like protein) as a predicted target of mtr-miR5741a (see **Supplementary Dataset 3**). Within the alignment-based approach, mtr-miR2589, mtr-miR482-5p, mtr-miR5287b, and mtr-miR319d-5p were predicted to target two methyltransferases (Medtr6g047800, Medtr5g079860), the CDC48 (Medtr7g088980), and DCD genes (Medtr4g084080) (see **Table 2** and **Supplementary Table 2**). All these hits bring an added value for plant science as they associate specific, previously unknown, miRNAs to the regulation of DDR functions. To date, there are only a few reports predicting DDR-associated functions as putative targets of miRNAs; for instance, MRE11 (Meiotic Recombination 11, a DSB repair nuclease) has been predicted as target of miR5261 in *Citrus sinensis* (Liang et al., 2017), or XPB2 (*Xeroderma pigmentosum* type B, an excision

repair helicase) predicted as target of miR122c-3p in *Triticum aestivum* (Sun et al., 2018). In human cell research, miRNAs involvement in the regulation of DDR is much more advanced and it is associated with the development of new therapeutic/diagnostic tools (Hu and Gatti, 2011; He et al., 2016). A number of studies document that p53, the master-regulator of DDR, is targeted by endogenous miRNAs (Hu et al., 2010; Kumar et al., 2011). This is the case of miR-25 and miR-30d, associated with p53 downregulation along with the suppression of downstream interactors p21, BAX, and Puma, hence being involved in apoptotic processes (Kumar et al., 2011). Another example, in relation to DNA repair pathways, indicates that hsa-miR-526b targets the Ku80 mRNA, with subsequent alterations of DSB repair and cell cycle arrest (Zhang et al., 2015). Our bioinformatics approach also revealed mtr-miRNAs predicted to target human genes with roles in DNA repair and related processes (see **Table 4**). To reiterate some examples, PPP4C (putatively targeted by mtr-miR169d-3p) catalyses the dephosphorylation of RPA2 (Replication Protein A2) in response to DNA damage, thus permitting the recruitment of RAD51 (an essential recombinase for the HR repair) to the damaged site (Lee et al., 2010). Likewise, FOXM1 (Forkhead Box M1), predicted as a target of mtr-miR169k, is among the most overexpressed oncoproteins in many types of cancer and therapeutic interventions to suppress its function are of great interest (Halasi et al., 2018). Hence, the identification of a miRNA belonging to conserved plant miRNA families (in this case, miR169) as a putative target of this gene may bring further support to ongoing cancer remedies. In relation to this, also many of the predicted targets associated with apoptosis (see **Table 5**), like the presented example of NOTCH1 (putatively targeted by mtr-miR5266), could have similar implications. The use of plant miRNAs as adjuvants in cancer therapies has been already tested; for example, plant miR159, abundantly found in human serum, has been associated with reduced incidence and progression of breast cancer because it targets the TCF7 (a Wnt signaling transcription factor) gene, causing decreased levels of MYC (Avian Myelocytomatosis Viral Oncogene Homolog) proteins, essential for cell cycle progression (Chin et al., 2016).

In conclusion, the current study provides comprehensive datasets (obtained by combining *ad-hoc* bioinformatics tools) related to *M. truncatula* miRNAs potential to putatively target genes across evolutionary distant species. By focusing on specific DDR-related functions, the hereby presented results significantly contribute to enriching the current knowledge regarding the conservation of DDR in plant and human cells. Considering the implications that some of these putative interactions could have for the biomedical sector, this study also offers additional hypotheses to be further experimentally validated. The developed pipeline can be applied to any species of interest to address species-specific cross-kingdom interactions or to carry out large-scale investigations involving a number of plant/animal species. The application of the proposed methods to other case-studies should take into account the following considerations on data, software, and knowledge availability: *(i)* the miRNAs of a 'donor' organism (e.g., a plant) and the transcriptome of the 'donor' and 'receiving' (e.g., an animal) organism should be available from public datasets or *de-novo* sequencing, annotation and expression studies; *(ii)* the miRNA dataset could be further refined by selecting experimentally known or computationally predicted miRNAs that are protected from degradation during incorporation in the receiver organism. The prediction miRNA targets in both species can be carried out *via* bioinformatic tools online available, although tool specificity for the target species should be taken into account and the parameter(s) of the algorithms should be fine-tuned accordingly, in order to have a balanced number of targets to be analysed in both species. Other target prediction algorithms may be used to overcome the so far weak knowledge on cross-kingdom regulation mechanisms to identify the target transcripts of heterologous miRNAs. For the miRNA target network reconstruction, an homology-based strategy should exploit the homology between the desired organisms and their model organisms in the same kingdom (e.g., *A. thaliana* for plants); on the other hand, a *de-novo* organism-specific co-expression network reconstruction relies on the availability of gene expression data from public datasets (e.g., GEO) or novel microarray/RNAseq experiments. All the networks can be analysed *via* specific software (e.g., Cytoscape) to find clusters of co-expressed genes and to carry out enrichment analyses on the desired gene sets. The experimental validation of the predicted targets can be subsequently performed *via* degradome analysis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MB, AM, and LP conceptualized the study. AM and LP wrote the manuscript. DM performed the network-based approach, MB performed the alignment-based approach, and ES performed the *M. truncatula* network reconstruction and analysis. AM, MB, and CG analysed the generated data. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01535/full#supplementary-material

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Exploring microRNA Signatures of DNA Damage Response Using an Innovative System of Genotoxic Stress in *Medicago truncatula* Seedlings

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One of the challenges that living organisms face is to promptly respond to genotoxic stress to avoid DNA damage. To this purpose, all organisms, including plants, developed complex DNA damage response (DDR) mechanisms. These mechanisms are highly conserved among organisms and need to be finely regulated. In this scenario, microRNAs (miRNAs) are emerging as active players, thus attracting the attention of the research community. The involvement of miRNAs in DDR has been investigated prominently in human cells whereas studies in plants are still scarce. To experimentally investigate the involvement of plant miRNAs in the regulation of DDR-associated pathways, an *ad hoc* system was developed, using the model legume *Medicago truncatula*. Specific treatments with camptothecin (CPT) and/or NSC120686 (NSC), targeting distinct components of DDR, namely topoisomerase I (TopI) and tyrosyl-DNA phosphodiesterase 1 (TDP1), were used. Phenotypic (germination percentage and speed, seedling growth) and molecular (cell death, DNA damage, and gene expression profiles) analyses demonstrated that the imposed treatments impact DDR. Our results show that these treatments do not influence the germination process but rather inhibit seedling development, causing an increase in cell death and accumulation of DNA damage. Moreover, treatment-specific changes in the expression of suppressor of gamma response 1 (*SOG1*), master-regulator of plant DDR, were observed. Additionally, the expression of multiple genes playing important roles in different DNA repair pathways and cell cycle regulation were differentially expressed in a treatment-specific manner. Subsequently, specific miRNAs identified from our previous bioinformatics approaches as putatively targeting genes involved in DDR processes were investigated alongside their targets. The obtained results indicate that under most conditions when a miRNA is upregulated the corresponding candidate target gene is downregulated, providing an indirect evidence of miRNAs action over these targets. Hence, the present study extends the present knowledge on the information available regarding the roles played by miRNAs in the post-transcriptional regulation of DDR in plants.

Keywords: DNA damage response, microRNA, genotoxicity, camptothecin, NSC120686, tyrosyl-DNA phosphodiesterase 1, seedling development

INTRODUCTION

During their lifespan, plants continuously face stressful conditions (caused by exogenous and endogenous factors) that affect plant growth and development. Considering their sessile lifestyle, plants are provided with incredible genomic plasticity. For instance, the metaphorical "perceptron," defined as an information-processing system composed of several processing units with biochemical connections, enables the selection of the most suitable options for coping with the changing environment (Scheres and Van der Putten, 2017). Linked to this, DNA damage response (DDR) is among the main strategies used by plant cells to safeguard their genome and therefore plant growth and development. To briefly define it, DDR is an intricated signal transduction network involving many players that act as DNA damage sensors, signal transducers, mediators, and effectors, working together to coordinate appropriate responses depending on the type of stimuli. A recent bibliometric study illustrates that DDR is generally far less studied in plants as compared to mammals but the interest in plant DDR research is expanding in view of future agricultural applications (Gimenez and Manzano-Agugliaro, 2017). Coincidently, it is also opportune to pinpoint that DDR is an evolutionarily conserved pathway in eukaryotes, although kingdom-specific variations are encountered (see reviews by Yoshiyama et al., 2013a; Nikitaki et al., 2018; Nisa et al., 2019). Just to cite some differences, suppressor of gamma response 1 (SOG1) is the proposed master-regulator of plant DDR, acting as a functional homolog of the mammalian p53 (Yoshiyama et al., 2009, 2013a,b). As a transcription activator, SOG1 regulates the expression of DNA repair- and cell cycle-related genes (Bourbousse et al., 2018; Ogita et al., 2018). Besides, SOG1-independent pathways have been also proposed to work in plant DDR; though the molecular mechanism is not yet fully understood, it is believed that these may include the E2F-RBR1 (RetinoBlastoma Related 1) complex, comprising transcription regulators that control the entry in the S-phase of the cell cycle (Berckmans and De Veylder, 2009). The E2Fa transcription factor also participates in DNA replication and DNA damage repair (Roa et al., 2009; Gutzat et al., 2012).

Ultimately, DDR enables the activation of cell cycle checkpoints as well as specific DNA repair mechanisms (Hu et al., 2016). Hence, the recognition and repair of DNA damage involve both the activation of DNA repair processes as well as the regulation of the cell cycle arrest, allowing the necessary time for DNA lesions to be corrected prior to cell cycle initiation. If DNA repair processes are impaired, changes in the cell cycle, transcription, and protein synthesis are encountered as well (Britt, 1999; Bray and West, 2005). Among the DNA damage repair mechanisms, some are highly specialized for specific types of damage whereas others work in a more generalized manner. It is also important to recognize that different DNA repair pathways have overlapping functions and can share key enzymes. For instance, Tyrosyl-DNA-phosphodiesterase 1 (TDP1), involved in the removal of topoisomerase I (TopI)-DNA covalent complexes (Yang et al., 1996; Pouliot et al., 1999), has been associated with both base excision repair (BER; Lebedeva et al., 2011; Donà et al., 2013) and DNA-protein crosslink (DPC) repair (Enderle et al., 2019a,b). Studies in model plants like *Arabidopsis thaliana* and *Medicago truncatula* showed that the lack of TDP1 function led to the development of dwarf genotypes sensitive to DNA damage with impaired DNA repair and cell cycle activities (Lee et al., 2010; Kim et al., 2012; Donà et al., 2013; Sabatini et al., 2016). The presence of a small subfamily of *TDP1* genes (composed of *TDP1α* and *TDP1β*) was highlighted in plants and it has been shown that the two genes do not have overlapping functions and they are differentially expressed in a species-, tissue-, and stress-specific manner (Macovei et al., 2010; Donà et al., 2013; Sabatini et al., 2017; Mutti et al., 2020).

To take place properly, the DDR system requires advanced regulatory mechanisms, which are not yet fully understood. In this context, microRNAs (miRNAs), a class of small, non-coding RNAs (~21–22 nt) that play key regulatory roles in gene expression at a post-transcriptional level (Bartel, 2004), may participate in the regulation of DDR. This aspect is quite recent and insufficiently explored, especially within the plant kingdom. Studies in human cells demonstrated that miRNAs are involved in the regulation of different components of the DDR machinery (Zhang and Peng, 2015). For instance, miR-24, miR-138, miR-182, miR-101, miR-421, miR-125b, and miR-504 were identified as crucial regulators of H2AX, BRCA1, ATM, or p53. Other such examples include miR-96, miR-155, miR-506, miR-124, miR-526, and miR-622b, shown to be involved in homologous recombination (HR) or nonhomologous end-joining (NHEJ) repair by targeting RAD51 or KU70/80 (Choi et al., 2014; Thapar, 2018). The presence of DNA lesions influences miRNA degradation as well as their expression. In both plants and animals, it has been demonstrated that miRNAs are responsive to irradiation (IR)-induced oxidative stress and may be responsible for the post-transcriptional regulation of some DDR genes (Joly-Tonetti and Lamartine, 2012; Kim et al., 2016). Plant specific miRNAs responsive to genotoxic stress include the IR-induced Arabidopsis miR840 and miR850, which remain to be further characterized in terms of their roles in DDR and DNA repair (Kim et al., 2016). Few rice miRNAs (osa-miR414, osa-miR164e, and osa-miR408) demonstrated to target specific helicases (Macovei and Tuteja, 2012) were also found to be responsive to γ-irradiation (Macovei and Tuteja, 2013). Predictive studies were employed as well; Liang et al. (2017) reported that MUTL-homolog 1 (MLH1) and MRE11 were putatively targeted by miR5176 and miR5261 in *Citrus sinensis* whereas the *Brachypodium distachyon* novel_mir_69 was identified to putatively target the RAD50 mRNA (Lv et al., 2016). Based on recent reviews of literature, an interrelation between DDR, redox systems, and miRNAs, has been proposed (Cimini et al., 2019). Nonetheless, specific hurdles have been pinpointed to explain the poorly represented examples in plants. Namely, this may be because DDR is significantly less studied in plants compared to animals (probably due to plant genome complexity) combined with limited information on miRNA targets specifically involved in coping with genotoxic stress (Chowdhury and Basak, 2019).

Considering the implications of DDR in plant genome stability, it is worth investigating deeper these fine-tuning aspects to gain novel insights on this complex topic. To address the existing gaps-of-knowledge, the current study proposes to explore the role of post-transcriptional regulation mediated by miRNAs in plant DDR. To do so, the first step consisted of setting up an original experimental system. This involved the administration of two compounds, namely, camptothecin (CPT, a well-known inhibitor of TopI enzyme) and NSC120686 (2-chloro-6-fluorobenzaldehyde 9H-fluoren-9-ylidenehydrazone). The latter was identified by Weidlich et al. (2010) as a substrate mimetic of the human TDP1. Together with topoisomerase inhibitors, NSC120686 has been used as a pharmacophoric model to suppress the TDP1 activity as part of a synergistic treatment for cancer therapies (Perego et al., 2012) whereas, in plants, dose-dependent genotoxicity was evidenced (Macovei et al., 2018a). As an experimental model, we have chosen to work on *M. truncatula*, because it is emerging as an informative and versatile system to investigate DDR during seed germination (Macovei et al., 2019). Moreover, DDR is an essential component of the seed repair response during germination (Waterworth et al., 2019) when active cell proliferation is determinant for the development of healthy seedlings and DNA damage must be repaired before the start of cell division to ensure the generation of robust plants. Phenotypic (germination percentage and speed, seedling growth) and molecular (cell death, DNA damage, and gene expression profiles) analyses demonstrated that the imposed treatments impact DDR. Subsequently, a list of miRNAs and putative target genes identified in a previous bioinformatics approach as being involved in DDR-associated biological processes (Bellato et al., 2019), were investigated in the developed system in terms of expression profiles. The results hereby presented show that miRNA/target gene expression is treatment-specific, thus confirming that miRNAs can be affected by DNA damage and that their targeted genes may have a contribution in the response to DNA damage.

MATERIALS AND METHODS

Experimental Design

Medicago truncatula (cv. Jemalong) seeds, kindly provided by Fertiprado L.d.a. (Portugal), were used for this study. Seeds were treated with 25 μM CPT (Sigma-Aldrich, Milan, Italy), and 25 μM NSC120686 (NSC) provided by the National Cancer Institute (Bethesda, United States). A combined CPT + NSC treatment was implemented as well. The concentrations of the genotoxic agents were selected based on preliminary phenotypic results (**Supplementary Figure S1**) and previous studies (Macovei et al., 2018a). Because these compounds are dissolved in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, Italy), specific DMSO controls, corresponding to each concentration used in the indicated treatments, were included. Specifically, DMSO 0.29% (subsequently denominated as DMSO_C) corresponds to the concentration used for the CPT treatments, DMSO 0.17% to NSC treatments (DMSO_N), and DMSO 0.23% to CPT + NSC treatments (DMSO_CN). The DMSO concentrations differ for CPT and NSC because the stock solutions (compounds dissolved in 100% DMSO) were prepared at different molarities (CPT 8.61 M and NSC 14.71 M), according to the manufacturer's instructions. This affected also the combined treatment, where CPT and NSC were mixed 1:1. A non-treated control (NT) was used for all experiments. The designated treatments were applied to *M. truncatula* seeds placed in Petri dishes (30 seeds per dish) containing a filter of blotting paper moistened with 2.5 ml H_2O (NT) or indicated solutions. Each sample/treatment was performed at least in triplicates. Petri dishes sealed with parafilm were kept in a growth chamber at 22°C under light conditions with a photon flux density of 150 μmol $m^{-2}s^{-1}$, photoperiod of 16/8 h, and 70–80% relative humidity. The experiment was followed for 7 days and subsequently, the harvested plant material was used fresh or frozen in liquid nitrogen (N_2) for designated analyses.

Phenotypic Evaluation

Treated and non-treated *M. truncatula* seeds were monitored for 7 days and data concerning germination percentage (%) and speed (T_{50}), seedling length, and fresh weight (FW) were determined at the end of the experiment. The germination % parameter was assessed as the percentage of total germinated seeds in which the radicle protrusion reaches at least 1 mm of length. The time required for 50% of seeds to germinate (T_{50}) was calculated according to the formula developed by Farooq et al. (2005): $T_{50} = t_i + [(N/2 - n_i)\ (t_i - t_j)]/n_i - n_j$, where N is the final seed germination, n_i, n_j represent the cumulative number of seeds that germinated by adjacent counts at times t_i and t_j when $n_i < N/2 < n_j$. Seedling length (millimeters, mm) was measured using millimetric paper whereas FW (grams, g) was measured using an analytical weight scale (Mettler AJ100, Mettler Toledo, Germany). Data are represented as mean ± SD of at least three independent measurements.

Single Cell Gel Electrophoresis

The single cell gel electrophoresis (SCGE) protocol was implemented to *M. truncatula* radicles as previously described (Pagano et al., 2017; Araújo et al., 2019). Nuclei were extracted from treated/untreated radicles isolated from freshly harvested 7-day-old seedlings. For nuclei extraction, liquid N_2 frozen radicles in Tris HCl EDTA (0.4 M Tris HCl pH 7.0, 1 mM EDTA pH 8) were finely sliced. The solution containing extracted nuclei was mixed with 1% low melting point (LMP) agarose and pipetted onto glass slides previously coated with 1% LMP. For alkaline SCGE, the glass slides containing isolated nuclei were subjected to electrophoresis (25 V, 300 A) in an alkaline buffer (0.3 M NaOH, 1 mM EDTA, and pH > 13) for 25 min at 4°C. For neutral SCGE, the slides were subjected to electrophoresis (20 V, 10 mA) in Tris-borate-EDTA (TBE; 89 mM Tris Base, 89 mM Boric Acid, 2 mM EDTA, and pH 8.3) for 8 min at 4°C. Subsequently, the slides were washed twice with Tris-HCl pH 7.5 for 5 min and rinsed in 70%

ethanol (v/v) for 12 min. For nuclei count, the slides were stained with 20 µl 4',6- diamidino-2-phenylindole (DAPI, 1 µgml^{-1} stock solution; Sigma-Aldrich) and visualized at a fluorescence microscope (Olympus BX51, Olympus, Germany) with an excitation filter of 340–380 nm and a barrier filter of 400 nm. For each slide, about 100 nuclei were scored and analyses were performed in triplicates. The results were expressed in arbitrary units (a.u) calculated according to the formula proposed by Collins (2004): $[\Sigma(N_c \times c) \times 100]/N_{tot}$, where N_c is the number of nuclei of each class, c is the class number (e.g., 0, 1, 2, 3, and 4), and N_{tot} is the total number of counted nuclei.

DNA Diffusion Assay

The DNA diffusion assay was performed to evaluate cell death events and distinguish cells subjected to PCD or necrosis from viable cells as described by Macovei et al. (2018b). Nuclei extraction was performed from radicles of 7-day-old seedlings using the same methodology described for SCGE. The glass slides containing nuclei embedded in 1% LMP agarose were incubated in high salt lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, and pH 7.5) for 20 min at 4°C to disrupt the nuclear membrane and permit DNA diffusion. The slides were immersed in neutral TBE for 5 min for three consecutive times to remove lysis solution and rinsed in 70% ethanol for 5 min at 4°C. Following DAPI staining, about 100 nuclei were scored (in triplicate samples) under the fluorescent microscope. The overall cell death level is given as a.u. while an additional analysis was used to represent the percentage of each class of nuclei (0-nuclei from viable cells, 1-nuclei from PCD cells, and 2-nuclei from necrotic cells).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was isolated from treated and untreated *M. truncatula* seedlings as described (Pagano et al., 2017; Araújo et al., 2019). Briefly, liquid N_2 grinded material was mixed with 550 µl Extraction Buffer (0.4 M LiCl, 0.2 M Tris pH 8.0, 25 mM EDTA, and 1% SDS) and 550 µl chloroform. Samples were centrifuged at 10,000 rpm for 3 min at 4°C. A phenol-chloroform solution was added to the supernatant followed by same centrifuge step. A 1/3 volume of 8 M LiCl was added to the supernatant, incubated at 4°C for 1 h, and subsequently centrifuged. The resulting pellet was washed with 70% ethanol, air-dried, and suspended in diethylpyrocarbonate (DEPC) water. The RNA was subsequently treated with DNase (Thermo Scientific), as indicated by the manufacturer. Finally, RNA was quantified with a NanoDrop spectrophotometer (Biowave DNA, WPA, ThermoFisher Scientific).

The complementary DNAs (cDNAs) were obtained using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's suggestions.

The quantitative real-time PCR (RT-*q*PCR) reactions were performed with the Maxima SYBR Green qPCR Master Mix (2X; ThermoFisher Scientific) according to the supplier's indications, using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics Pty Ltd., Brisbane, Queensland Australia). Amplification conditions were as follows: denaturation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s. Oligonucleotide primers (**Supplementary Table S1**) were designed using Primer3Plus[1] and verified with Oligo Analyzer.[2] The relative quantification was carried out using actin-related protein 4A (*Act*) and elongation factor 1α (*ELF1α*) as reference genes since they resulted the most stable under the tested conditions following geNorm (Vandesompele et al., 2002) analysis (**Supplementary Figure S2**). The raw, background-subtracted fluorescence data provided by the Rotor-Gene 6000 Series Software 1.7 (Corbett Robotics) was used to estimate PCR efficiency (E) and threshold cycle number (C_t) for each transcript quantification. The Pfaffl method (Pfaffl, 2001) was used for the relative quantification of transcript accumulation. All reactions were performed in triplicate. The data are presented as fold change (FC), where values for each treatment were normalized to their corresponding DMSO control. Heatmaps were constructed using the Shinyheatmap tool (Khomtchouk et al., 2017).

microRNAs Expression Analysis

For miRNAs expression, total RNA was isolated using TRIzol (ThermoFisher Scientific), as indicated by the supplier. The two-tailed RT-*q*PCR technique (Androvic et al., 2017) was performed to quantify miRNA accumulation. The miRNAs expression profiles were analyzed in 7-day-old untreated and treated seedlings. Different sets of primers were used to perform reverse transcription (RT) and RT-*q*PCR for each mature miRNA, one to synthesize the cDNA and two for the SYBR *q*PCR amplification. cDNAs were obtained using the qScript® Flex cDNA Synthesis Kit (QIAGEN, Beverly, Massachusetts). The RT primers (**Supplementary Table S2**) were designed to have a two-tailed structure as indicated by Androvic et al. (2017). RNAfold WebServer[3] was used to predict the stable secondary structure. To obtain the cDNA, a forward primer specific for the designed region in the 5'-terminus of the two-tailed RT-primer and a reverse primer specific for the miRNA target sequence were used. Subsequently, RT-*q*PCR was performed as described in the above paragraph using the oligonucleotide primers shown in **Supplementary Table S3**.

Statistical and Integrative Data Analyses

For phenotypic evaluation, the significance of mean differences was determined using the Student's *t*-test. For gene/miRNA expression data, following the normality test (Shapiro-Wilk), a one way ANOVA on ranks was performed using the Kruskal-Wallis test in an R (software version 4.0.2) background.

Principal components analysis (PCA) was performed on the phenotypic and molecular variables quantified across the study using the FactoMineR (Lê et al., 2008) and factoextra (Kassambara and Mundt, 2020) packages in R environment for statistical computing and graphical design. Values were

[1]https://primer3plus.com/
[2]https://eu.idtdna.com/calc/analyzer
[3]http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi

standardized by means of *z*-score using the default scaling settings of the PCA function. The included variables were: germination %, T_{50}, seedling length (divided as aerial part and radicles), DNA damage levels, all gene expression data, and miRNA expression profiles.

RESULTS

CPT and NSC Treatments Do not Affect Seed Germination but Impair Seedling Development

The CPT and NSC120686 inhibitors require to be dissolved in DMSO, which, at certain concentrations, can impair plant development (Zhang et al., 2016). Thus, it was necessary to first identify the inhibitor concentrations at which minimal or null DMSO effects are evident at a phenotypic level. In the case of the CPT treatments, the selected concentration was 25 μM dissolved in 0.29% DMSO (**Supplementary Figure S1**). The selection of NSC concentration (25 μM dissolved in 0.17% DMSO) was based on previous results (Macovei et al., 2018a). The last treatment consisted of synergistically exposing *M. truncatula* seeds to CPT 25 μM and NSC 25 μM (treatment denominated as CPT + NSC), dissolved in 0.23% DMSO. As described in "Materials and Methods," each corresponding DMSO concentrations (denominated as DMSO_C, DMSO_N, and DMSO_CN) were tested along with the non-treated (water) control (NT).

To verify whether CPT and NSC influence seed germination, a phenotypic characterization was performed by evaluating germination % and speed (T_{50}), seedling length, and FW after 7 days of treatment. While seed germination % and T_{50} were not significantly affected by any of the imposed treatments at the end of the indicated timeframe (**Supplementary Figure S3**), CPT impacted mostly on seedling development. **Figure 1A** shows the morphology of the 7-day-old seedlings, grown in the presence of CPT, NSC, and CPT + NSC, and their corresponding DMSO controls. Treatment with the NSC inhibitor did not result in a visible change in seedling morphology while seedlings treated with CPT and CPT + NSC appeared shorter and stockier than the relative controls. These observations are supported by the registered significant ($p < 0.05$) differences when measuring the

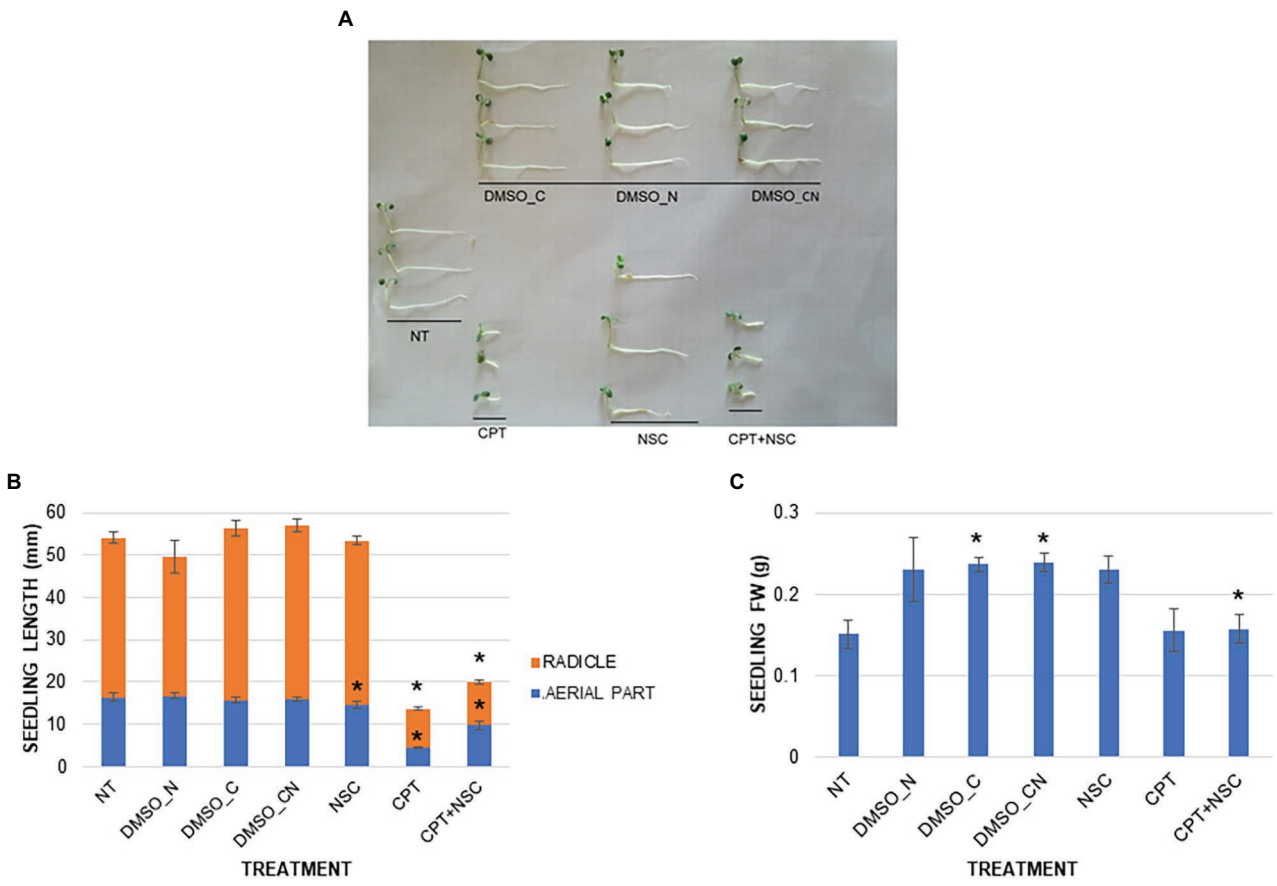

FIGURE 1 | Phenotypic effect of CPT, NSC, and CPT + NSC treatments, and corresponding DMSO concentrations (DMSO_N, DMSO_C, and DMSO_CN) on *Medicago truncatula* seed germination. **(A)** Representative image of 7-day-old seedlings. **(B)** Seedling length (mm). **(C)** Fresh weight, FW (g). Data are represented as mean ± SD of three independent replicates. Statistically significant ($p < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; and DMSO, dimethyl sulfoxide.

seedling length and FW (**Figure 1B**). A reduction in seedling length was caused by the CPT and CPT + NSC treatments, with radicles being more severely affected than the aerial parts. A minor, although still significant impact, was observed in the case of the NSC-treated seedlings. When considering the FW parameter, an increase in seedling weight was detected for DMSO_C and DMSO_N, while FW was significantly decreased in the NSC + CPT-treated seedlings (**Figure 1C**).

Overall, these results show that the imposed treatments do not affect germination *per se* but inhibit seedling growth, especially in the CPT- and CPT + NSC-treated samples. This may lead to assume that CPT contributes the most to the impairment of the seedling growth since a lesser effect was observed when the NSC compound was delivered alone.

The Imposed Treatments Induce Different Cell Death Profiles

A DNA diffusion assay was performed to evaluate cell death events in 7-day-old *M. truncatula* seedlings subjected to CPT and NSC treatments (**Figure 2**). The results of the diffusion assay were expressed both as arbitrary units (a.u.) to indicate the overall level of cell death and as percentage of nuclei per class to indicate the different types of cell death events (class 0 – viable cells, class 1 – programmed cell death events, and class 2 – necrosis events). Enhanced levels of cell death are evident in the imposed treatments when compared to NT, with the highest values registered during the CPT treatment (**Figure 2A**). Cell death significantly increased also in samples treated with DMSO_C and DMSO_CN but at a substantially lesser degree than when compared to the CPT/NSC system. When looking at the different types of nuclei classes, the data show that the NT and DMSO_N samples are both characterized by a high percentage of viable nuclei (86.36 ± 6.00 and 83.63 ± 3.16%, respectively) and a low percentage of PCD and necrosis (**Figure 2B**). Seedlings treated with DMSO_C and DMSO_CN started to show a decrease in viable nuclei (47.60 ± 3.40, 55.74±4.74%) toward PCD, while the nuclei classified as belonging to necrotic cells (class 2) are not present.

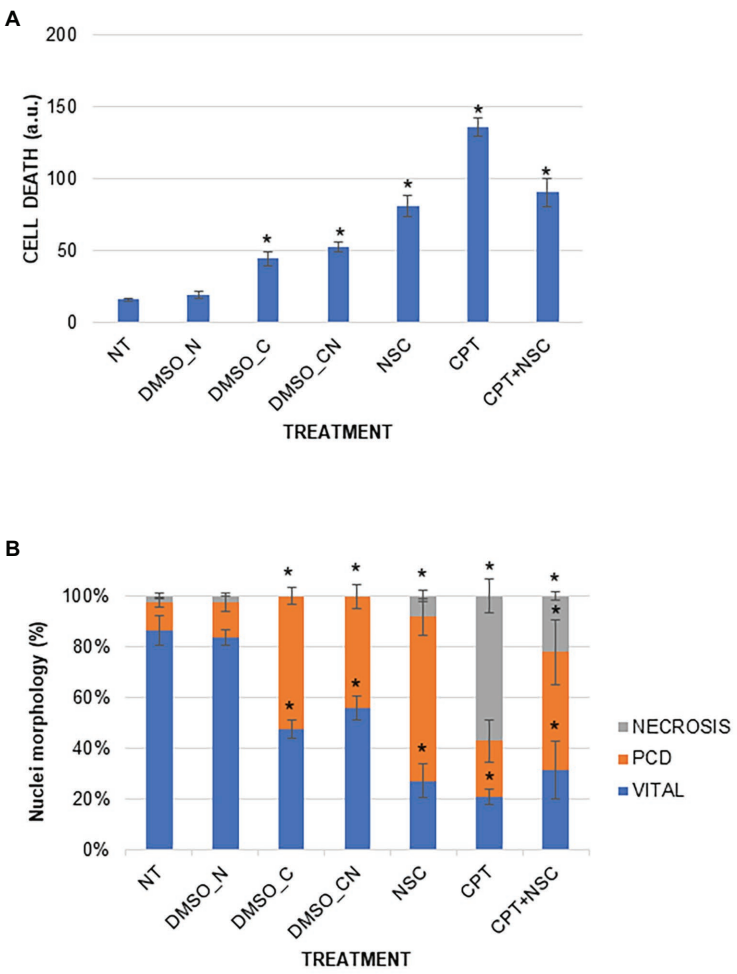

FIGURE 2 | Cell death induced by the imposed treatments in *M. truncatula* 7-day-old seedlings. **(A)** Overall cell mortality scores represented as arbitrary units (a.u.). **(B)** Cell death represented as percentage of nuclei per class. Values are expressed as mean ± SD of three replicates. Statistically significant ($p < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; and DMSO, dimethyl sulfoxide.

Class 2 nuclei are mostly present in CPT and CPT + NSC samples, while the NSC treatments evidence the presence of class 1 nuclei characteristic for PCD events (**Figure 2B**). Concerning the NSC- and CPT + NSC-treated samples, a marked decrease in the percentage of viable nuclei (27.18 ± 6.76, 31.52 ± 11.18%) is observed with a concomitant increase in the percentage of nuclei subjected to PCD (52.12 ± 5.49, 46.53 ± 12.7%) and necrosis (27.38 ± 6.20, 21.9 ± 6.20%). Similarly, a reduction in the percentage of viable nuclei is observed for CPT-treated samples (21.05 ± 2.91%), where the most represented nuclei belong to class 2 (57.13 ± 6.82%), characteristic for the presence of necrotic events.

Overall, the imposed treatments decrease cell vitality and induce different types of cell death events. The most severe effects are observed with the CPT treatment, characterized by a high level of necrosis whereas PCD events prevail in the NSC treatment. In the CPT + NSC combination, both PCD and necrosis events are registered at similar levels.

The Imposed Treatments Cause Accumulation of DNA Damage

To quantitatively measure DNA damage, SCGE was performed using both the alkaline and neutral versions of the assay. Representative images for each nuclei class (0–4) are provided (**Figure 3A**). The neutral version generally detects double-stranded breaks (DSBs) whereas the alkaline version includes different types of breaks such as single-strand breaks (SSBs) formed from alkali-labile sites, DNA-DNA, or DNA-protein cross-links (Ventura et al., 2013). Compared to NT, the NSC-treated samples showed a 7.22-fold increase in the level of DNA damage under alkaline conditions while only a 1.99-fold increase was observed under neutral conditions (**Figure 3B**). A 5.86- and 5.79-fold increase in the level of DNA damage was observed in the CPT-treated samples under alkaline and neutral conditions, respectively. The CPT + NSC-treated samples showed a 13.7-fold increase in the level of DNA damage in alkaline conditions while an 8.4-fold increase was detected under neutral conditions. Considering the DMSO controls, no significant differences in the accumulation of DNA damage as DSBs are evident under neutral conditions. However, a small but significant increase in the levels of DNA damage was registered under alkaline conditions. This may suggest that DMSO could generate SSBs, alkali-labile sites, incomplete excision repair sites, and DNA-DNA/DPCs rather than more extensive damage like DSBs.

Overall, the observed results indicate that the administration of CPT/NSC agents cause an accumulation of both SSBs and DSBs, but at different degrees depending on the type of treatment. While in the case of NSC, SSBs and associated damage types are prevalent, for the CPT treatments an additional increase in the presence of DSBs is observed. The combination of the two agents (CPT + NSC) resulted in the highest level of DNA damage, combining DSBs, SSBs, and associated damage, the latter being most prevalent.

CPT/NSC Treatments Trigger Differential Expression of DDR-Related Genes

Given that CPT/NSC treatments resulted in reduced seedling growth, increased cell mortality, and accumulation of DNA damage, the next step consisted in the evaluation of DDR-related gene expression profiles using RT-*q*PCR. The following genes were selected:

1. *SOG1*, as the master-regulator of plant DDR;
2. *TDP1α*, *TDP1β*, *TDP2α*, *Top1α*, and *Top2*, as genes that encode for proteins most probably affected by the CPT and NSC inhibitors;
3. *MRE11*, *RAD50*, *NBS1*, *PARP1*, *ERCC1*, and *MUS81*, as genes that encode for proteins involved in repair processes considered as alternative to the function of *TDP1* genes. The genes belonging to the MNR complex were selected as they represent the frontline players in the detection and

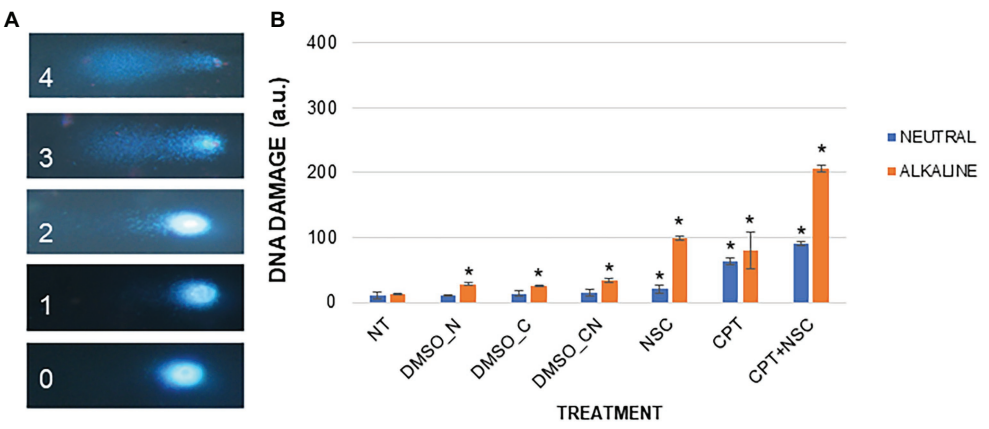

FIGURE 3 | DNA damage induced by the imposed treatments in *M. truncatula* 7-day-old seedlings. **(A)** Nucleus morphology and its related class identification number. **(B)** DNA damage scores represented as a.u. Values are expressed as mean ± SD of three replicates. Statistically significant ($p < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; and DMSO, dimethyl sulfoxide.

signaling of DSBs, thus HR and NHEJ repair pathways. On the other side, *PARP1*, *ERCC1*, and *MUS81* are associated with both BER and DPC repair. All selected genes were already validated in *M. truncatula* calli exposed to NSC120686 (Macovei et al., 2018a).

4. *TOR*, *CDKA1*, *CycB1*, *CycD2*, and *CycD3*, as genes that encode for proteins known to be involved in the regulation of the cell cycle.

Because the expression of the genes appears to be influenced by DMSO (**Supplementary Figure S4**), and to evaluate the real effect that CPT and NSC treatments may induce at the level of gene expression, the data are presented as FC to control, where the control is represented by each corresponding DMSO concentration. The FC values were used to generate a heatmap (**Figure 4**), where blue color indicates downregulated genes and red color indicates upregulated genes compared to their respective controls. The ANOVA analysis show statistical differences ($p < 0.05$) between treatments and controls for the majority of investigated genes (**Supplementary Table S4**). These results show that the *SOG1* gene is upregulated by CPT and downregulated by NSC, suggesting a contrasting effect of the two treatments at the level of DDR. This contrasting trend is maintained as well when looking at the expression levels of most investigated genes. *TDP1α*, *TDP1β*, and *TopIα* are upregulated by NSC and downregulated by CPT treatments. Conversely, most of the genes involved in alternative DNA repair pathways (*PARP1*, *ERCC1*, *MUS81*, *MRE11*, and *NBS1*) are upregulated by CPT and downregulated by NSC treatments. Within the genes involved in the regulation of the cell cycle, *Cdka1*, *Cycd3*, and *TOR* are upregulated during CPT treatments whereas *Cycb1* is upregulated by NSC. The concomitant administration of CPT + NSC had a different response compared to the individual CPT or NSC treatments; namely, most of the investigated genes are downregulated and the only upregulated genes are *TDP2α*, *MUS81*, and *Cycd2*.

Overall, the gene expression data indicate a contrasting effect for the single administration of NSC and CPT treatments along with a distinct response in case of the synergistic exposure to both compounds where most investigated genes appeared downregulated.

Expression Analyses of Selected microRNAs and Their Putative Targets

Since the main goal of this work was to identify miRNAs able to regulate DDR-associated processes, we proceeded with the investigation of different miRNA-target gene pairs, previously identified from bioinformatics analyses as being related to DDR processes (Bellato et al., 2019). The expression profiles of selected

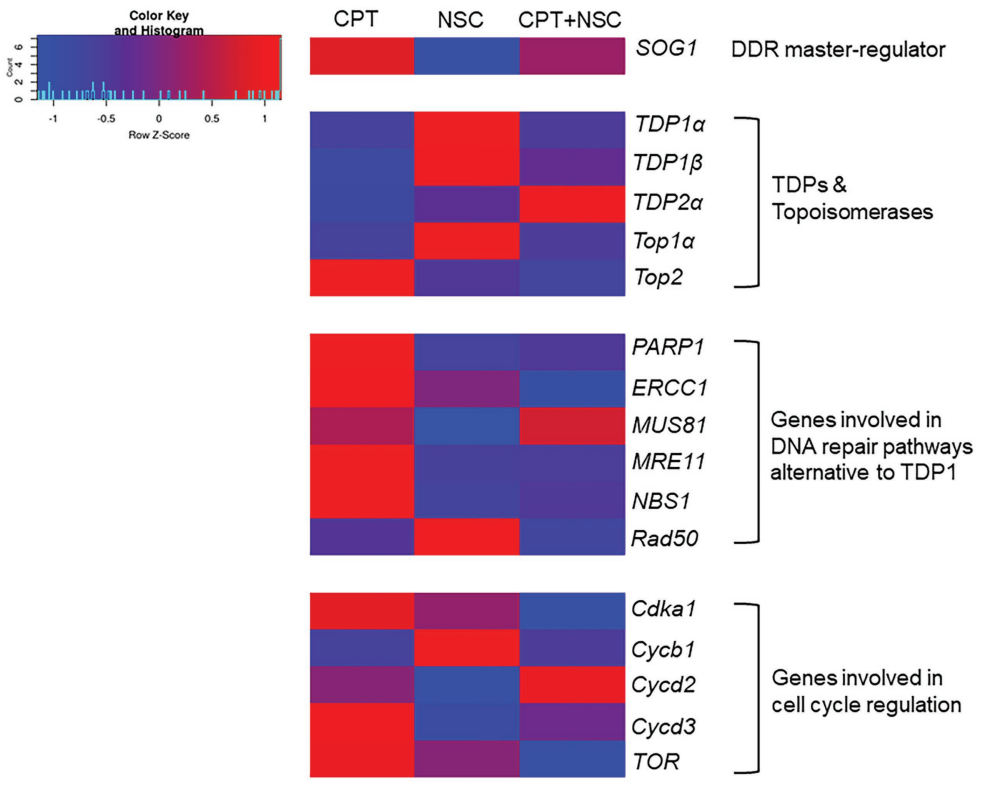

FIGURE 4 | Heatmap representing fold changes (FCs) in gene expression values in response to CPT, NSC, and CPT + NSC treatments in 7-day-old *M. truncatula* seedlings. For each treatment, the values were normalized to their corresponding DMSO controls. The heatmap was constructed using the Shinyheatmap. CPT, camptothecin; NSC, TDP1 inhibitor NSC120686 (http://shinyheatmap.com/) application.

miRNAs and putative target genes were investigated in the CPT/NSC system, proven to affect DDR. Specifically, the following miRNA-gene pairs were considered:

1. mtr-miR156a, identified as putatively targeting *UBE2A* (ubiquitin-conjugating enzyme, Medtr4g108080), involved in histone modification processes.
2. mtr-mir172c-5p, putatively targeting *RAD54-like* (DNA repair and recombination RAD54-like protein, Medtr5g004720), involved in DSBs repair.
3. mtr-miR2600e, putatively targeting *5AT* (anthocyanin 5-aromatic acyltransferase, Medtr2g089765), involved in antioxidant defense.
4. mtr-mir395e, putatively targeting *DMAP1* (DNA methyltransferase 1-associated protein, Medtr1g086590), associated with histone modifications.
5. mtr-miR5741a, putatively targeting *E2FE-like* (E2F transcription factor-E2FE-like protein, Medtr4g106540), involved in DNA-dependent DNA replication.
6. mtr-miR168a, targeting *AGO1A* (Argonaute protein 1, Medtr6g477980), used as a control since the relation between this miRNA and target gene has already been experimentally validated (Vaucheret et al., 2004, 2006).

The expression profiles of miRNAs and putative target genes are shown in **Figure 5** while associated statistics are given in **Supplementary Table S4**. First, their expression in non-treated (NT) samples was monitored to evaluate their behavior under physiological conditions. As shown in **Figure 5A**, while the majority of the tested miRNAs are highly expressed (except for mtr-miR395e), the expression of their putative target gene is significantly reduced, thus corroborating the expected trend where miRNAs activity inhibits the expression of the targeted gene. The ability of miR168a to target *AGO1A* gene is a well-known fact to the scientific community (Vaucheret et al., 2004, 2006), therefore, this miRNA was chosen as quality control for function/target validation. Indeed, a low level of *AGO1A* expression corresponds to a high level of miR168a expression in NT samples (**Figure 5A**). Looking into the expression of this specific miRNA and its target gene during the imposed treatments, it is evidenced that when the expression of miR168 is low, the expression of *AGO1A* is high, and vice-versa (**Figure 5B**).

Since gene expression is influenced by DMSO, also in this case, data are represented as FC to respective controls and gathered in a heatmap (**Figure 5C**) where blue color represents downregulation and red color represents upregulation. Overall, the heatmap shows that under most conditions when a miRNA is upregulated the corresponding candidate target gene is downregulated. Looking at the miRNAs expression according to each treatment, it is possible to observe a treatment-specific behavior where different miRNAs expression is triggered by different treatments. Namely, mtr-miR156a and mtr-miRA5671 are upregulated by CPT, mtr-miR172c-5p is upregulated by NSC, and mtr-miR2600e are upregulated by CPT+NSC.

Overall, an indirect evidence of miRNA action over these targets is provided; the contrasting profiles between miRNA-predicted target abundances support the evidence that these miRNAs could repress the expression of these targets.

Principal Component Analysis for Data Integration

Principal components analysis was used to investigate the differences between samples and which variables most contributed to these differences (**Figure 6**). The *X*-axis and *Y*-axis show the principal dimension Dim1 and Dim2 that explain 29.1 and 21.5% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. The orientation of the ellipses shows that the most different samples are those treated with CPT and CPT + NSC whereas the NSC treatment is located in the proximity of DMSO_CN- and DMSO_N-treated samples (**Figure 6A**). Other distinctive groups are formed by the NT and DMSO_C samples located in the upper-right panel. Hence, the plotted data allow a clear separation of the majority of the samples according to the imposed treatments. The observed vicinity among replicates is indicative of data reliability. The variables that most contributed to the group differentiation are represented in a light blue color (**Figure 6B**). Among the phenotypic parameters, the most representative variables include seedling length, cell death, and DNA damage. Amidst the investigated genes, DMAP1, E2FE-like, PARP1, Cycd3, and Cycd2 had the highest contribution but also TDP1β, Top1α, Top2, and NBS1 are well-represented. When considering the miRNAs, it is relevant to underline that these had an important contribution to the differentiation of the samples and the most representative ones are mtr-miR2600e and mtr-miR5741.

DISCUSSION

In this work, CPT and NSC120686 were used alone or in combination to develop an original experimental system in which plant specific DDR functions would be altered so that miRNAs associated with DDR pathways could be revealed. CPT is a widely used agent much employed in anticancer therapies due to its activity as TopI inhibitor since it intercalates between DNA breaks flanking the TopI-cleavage complex (Pommier et al., 2010). CPT is known for its cytotoxic effects also in plants (Buta and Worley, 1976; Takahashi et al., 2002) where enhanced levels of cell death had been registered (Locato et al., 2006; Iakimova et al., 2020) presumably through the accumulation of TopI-covalent complexes as in the case other eukaryotes. On the other side, the NSC120686 compound was recently identified based on virtual screening of pharmacophores able to inhibit human Tdp1 (Weidlich et al., 2010) and subsequently used in combination with CPT-derivates to inhibit the growth of different cancer cell lines (Perego et al., 2012). *Medicago truncatula* calli treated with different concentrations of NSC120686 displayed enhanced levels of cell mortality and DNA damage (Macovei et al., 2018a). So far, combined application of the two agents has not been reported in plants.

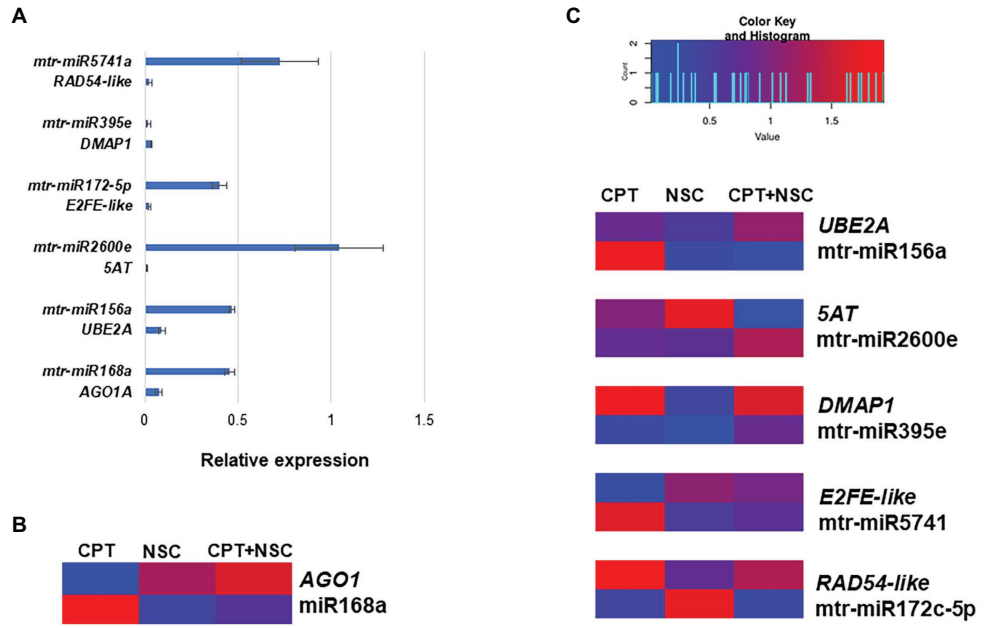

FIGURE 5 | Expression profiles of selected miRNAs and their putative targeted genes in 7-day-old *M. truncatula* seedlings. **(A)** Relative expression of miRNAs/genes pairs in non-treated (NT) samples. **(B)** *AGO1A* and mtr-miR168a pair used as data quality control. **(C)** Heatmap representing fold changes (FCs) to each corresponding DMSO of miRNAs and putative targeted genes in response to CPT, NSC, and CPT + NSC treatments. The heatmap was constructed using the Shinyheatmap (http://www.shinyheatmap.com/) application. CPT, camptothecin; NSC, TDP1 inhibitor NSC120686

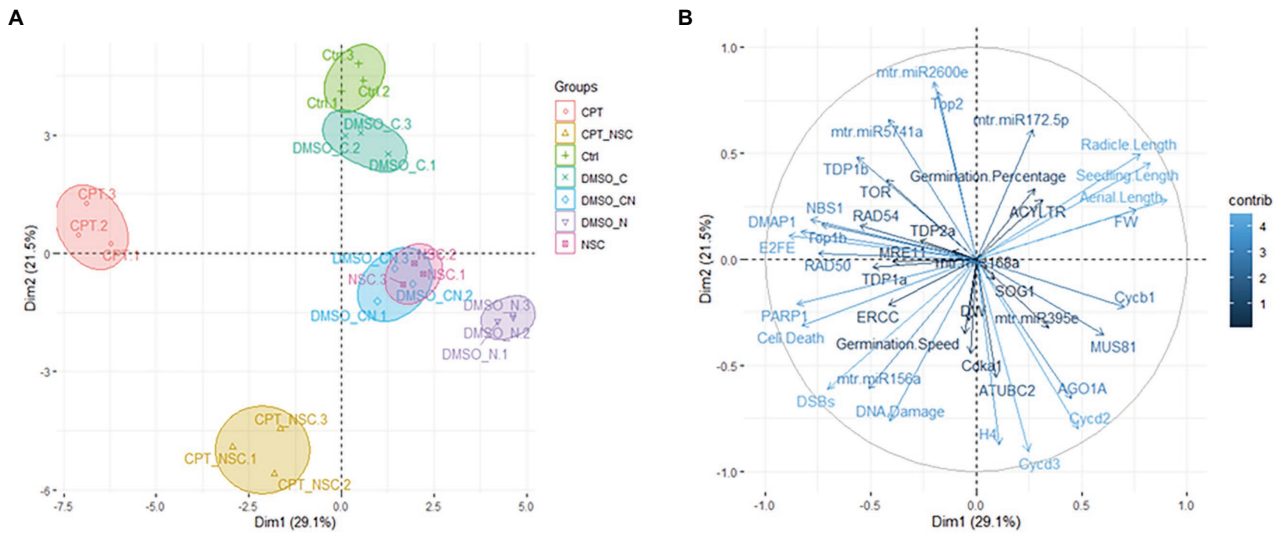

FIGURE 6 | Principal component analyses (PCA). **(A)** Loading plot explaining the distribution of samples focusing on the imposed treatments. **(B)** Loading plot explaining the contribution of each measured variable (germination %, T_{50}, seedling length, FW, gene, and miRNAs expression profiles).

Before evaluating the possible involvement of miRNAs in this system, it was first necessary to prove that it targets DDR-associated processes. The phenotypic investigation revealed that CPT and CPT + NSC had a major effect on seedling development mostly by inhibiting radicle growth while the single administration of NSC had a milder effect (**Figure 1**). Hence, the phenotypic changes could be mostly attributed to CPT, as in agreement with previous studies, where 25 μM of CPT substantially inhibited the growth of *Arabidopsis* plantlets while concentrations higher than 50 μM resulted

in a strong impairment of both roots and shoots at young seedling stages (Takahashi et al., 2002). In accordance with the observed phenotypes, enhanced levels of cell death and accumulation of DNA damage were evidenced (**Figures 2, 3**). Interestingly, different types of cell death events and DNA damage were encountered according to the imposed treatments. While CPT administration resulted in enhanced levels of necrosis and accumulation of DSBs, the delivery of NSC was accompanied by PCD and accumulation of SSBs, DNA-DNA, or DPCs. For the CPT + NSC combination, both PCD and necrosis events are present at similar levels while the high levels of DNA damage indicate the most genotoxic effect. Previous literature reports that CPT results in the accumulation of DPCs (Enderle et al., 2019b) and DSBs (Ferrara and Kmiec, 2004; Berniak et al., 2013), lesions know to be associated with necrotic events in plant cells (Rowan et al., 2010; Song and Bent, 2014). On the other hand, low concentrations of NSC120686 resulted in enhanced levels of PCD in *M. truncatula* calli (Macovei et al., 2018a).

The outlined distinction between treatments was maintained when considering the expression profiles of selective genes belonging to different DNA repair pathways and cell cycle regulation (**Figure 4**). In addition to *TDP1*, *α*, and *β*, and *Top1α* genes, *TDP2α*, and *Top2* genes were investigated because of the closed connection between these two, as TDP2 enzyme is involved in the removal of DNA TopII-mediated DNA damage and cell proliferation/differentiation signaling (Cortes Ledesma et al., 2009). Moreover, the overexpression of *TDP2α* gene in *M. truncatula* was correlated with a decrease in the accumulation of DSBs, increased cell proliferation, and enhanced resistance to stress (Confalonieri et al., 2014; Faè et al., 2014; Araújo et al., 2016). Genes involved in DNA repair alternative to TDP1 (Pommier et al., 2014) include the MNR complex, composed of *MRE11*, *NBS1*, and *Rad50*, known to be involved in the detection of DBSs and HR (Manova and Gruszka, 2015) as well as *PARP1*, *MUS81*, and *ERCC1* involved in BER and DPC repair (Enderle et al., 2019b; Roldán-Arjona et al., 2019). Since DDR includes a response from both DNA repair and cell cycle regulation, several cyclins (*Cdka1*, *Cycb1*, *Cycd2*, and *Cycd3*) were investigated alongside the master-regulators *TOR* and *SOG1*. The observed changes in the expression profiles of *SOG1* gene indicate that DDR is truly affected by the imposed treatments; hence, we can conclude that the developed system has an impact on DDR. To briefly summarize the behavior of the tested genes in association with the phenotypic observations, the following assumptions are taken into consideration (**Figure 7**):

1. During the CPT treatment, TopI enzyme is presumably blocked, TopI-DNA covalent complexes would accumulate and high levels of DNA damage and cellular mortality are registered, resulting in substantial inhibition of seedling growth. In this situation, *TDP1* and *Top1* genes are inhibited while genes involved in DNA repair pathways alternative to TDP1 are highly active. Based on the expression of genes involved in the cell cycle, this is delayed presumably to allow the repair of the induced DNA damage.

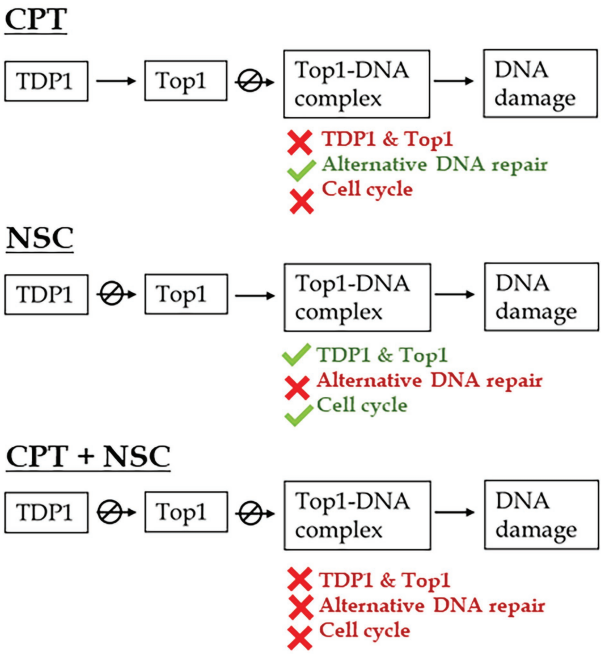

FIGURE 7 | Schematic representation of the proposed effects of CPT and NSC, inhibitors of Top1 and TDP1 enzymes, on DNA repair pathways and cell cycle regulation during *M. truncatula* early seedling development. CPT, camptothecin; NSC, TDP1 inhibitor NSC120686.

2. When NSC is given, the TDP1 enzyme would interact with this mimicking compound, thus being prevented from engaging with its substrate and hydrolyze the crosslink between TopI and DNA. In turn, this may again lead to the accumulation of these complexes and the subsequently observed enhancement in the levels of cell death and DNA damage, although at a lesser extent, in agreement with the phenotypic observations. In this case, the *TDP1* and *Top1* genes are active, the alternative DNA repair is inhibited, and the cell cycle is progressing.
3. The CPT + NSC combination may target both TDP1 and TopI functions and this leads to the highest cytotoxic and genotoxic effects, corresponding to the obstructed seedling development. In terms of gene expression, this treatment induced the downregulation of most of the investigated genes, affecting both DNA repair and cell cycle progression.

In a previous bioinformatics investigation, we have identified specific miRNAs (mtr-miR156a, mtr-mir172c-5p, mtr-miR2600e, mtr-mir395e, and mtr-miR5741) putatively targeting genes associated with DDR processes (Bellato et al., 2019). Among these, miR156 is an evolutionarily conserved family, although diversification in its members, sequence, and functions are present (Sunkar and Jagadeeswaran, 2008; Cui et al., 2017). Others, like miRNA172 family has been associated with seed development alongside with other regulatory functions (Smoczynska and Szweykowska-Kulinska, 2016). High-throughput sequencing of *M. truncatula* seedlings found

that miR156 and miR172 are involved in salinity stress (Cao et al., 2018). MiR395 is involved in sulfate assimilation regulatory network (Matthewman et al., 2012) whereas miR5741 has been associated with roles in the defense response (Siemens et al., 2006). It is therefore clear that these miRNAs have been studied mainly in relation to plant development and response to biotic/abiotic stress. The RT-*q*PCR analyses performed in this work indicate that they are also involved in the response to genotoxic stress, as indicated by their differential expression induced by the CPT/NSC treatments. For example, mtr-miR172c-5p is upregulated in NSC treated samples and downregulated in CPT treated samples. By observing the expression profiles of its putatively targeted gene *E2FE-like*, it is shown that an upregulation of the miRNA is accompanied by a downregulation of the gene predicted to be its target. Importantly, this gene is a homolog of the *Arabidopsis* E2F transcription regulator shown to be involved in cell cycle regulation, DNA replication, and DNA damage repair, in pathways alternative to SOG1 (Berckmans and De Veylder, 2009; Roa et al., 2009; Gutzat et al., 2012).

In conclusion, by inducing seedling growth inhibition, accumulation of cell death, and DNA damage, along with the differential expression of genes involved in DDR, the developed CPT/NSC system actively influence DDR-associated processes. Above all, we demonstrated that specific miRNA-target gene pairs, identified from a bioinformatics approach, are responsive to the imposed treatments, thus showing that these miRNAs have a role to play in DDR. This study extends the knowledge regarding the roles played by miRNAs in the post-transcriptional regulation of DDR in plants. This may disclose new regulatory networks with further possibilities regarding biotech application relevant to enhance crop adaptation to genotoxic stresses. Given the complexity of regulatory networks and since miRNAs can repress multiple targets, further functional validation studies are needed to corroborate these suggested roles in DDR. This is particularly relevant to clarify if other regulatory mechanisms might be responsible for the observed downregulation of target genes expression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AM conceptualized the study. CG, MG, AP, and TC performed the treatments and conducted the designed analyses. AM, MG, CG, and SA analyzed and interpreted the data. AM and AB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.645323/full#supplementary-material

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