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Differential Expression of AGO1a/AGO1b in Chilli in Response to Chilli Leaf Curl virus Infection

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ABSTRACT: RNA silencing, mediated by small interfering RNAs (siRNAs) and microRNAs (miRNAs), is a defensive pathway used by plants to combat invading nucleic acids. Argonaute (AGO) proteins play a central role in orchestrating this process and are involved in antiviral defense mechanisms. While the involvement of AGO1 in response to RNA viruses has been extensively studied, its expression levels in DNA virus infections remain understudied. In this study, we investigated the expression levels AGO1a and AGO1b genes in response to chilli leaf curl virus (ChiLCV) infection, a DNA virus that infects chilli plants at different days post virus inoculation. Semi-qPCR and qPCR analysis showed a significant increase in the expression levels of both AGO1a and AGO1b transcripts in response to ChiLCV infection compared to control plants. These findings provide valuable insights into the involvement of AGO1 proteins in the plant's defense against DNA viruses and expand our understanding of the RNA silencing pathway in antiviral responses. This study could be helpful in developing strategies for viral disease management of DNA viruses through modulating the levels or activity of AGO proteins and its interacting miRNAs.

Keywords: RNAi, Argonauts, chilli leaf curl virus, differential gene expression.

INTRODUCTION

RNA silencing is a defensive pathway that can be triggered to combat invading nucleic acids by utilizing small interfering RNAs (siRNAs). This process involves the specific targeting and deactivation of mRNA or transcripts through siRNAs or miRNAs with a sequence length ranging from 21 to 24 nucleotides. The mechanism behind this phenomenon was first described and experimentally validated by Fire et al. (1998) while studying *Caenorhabditis elegans*, leading to the coining of the term "RNA interference."The initial step of RNA interference (RNAi) involves the conversion of long double-stranded RNA (dsRNA) molecules into shorter RNA molecules known as small interfering RNAs (siRNAs) or microRNAs (miRNAs), which are typically 21-24 nucleotides in length. This processing is facilitated by an enzyme called Dicer, resulting in the formation of an siRNA or miRNA duplex with two nucleotide overhangs at the 3' end. Subsequently, the duplex interacts with the endonucleolytic protein called Argonaute (AGO) along with other associated proteins, leading to the assembly of an RNA-induced silencing complex (RISC) containing one of the sRNA strands. The RISC complex then proceeds to bind to messenger RNAs (mRNAs) that possess a perfectly complementary sequence to the sRNA strand. Upon binding, the Argonaute protein within the RISC complex cleaves the siRNA:mRNA or miRNA:mRNA duplex at the middle, resulting in the degradation of the targeted mRNA (Baulcombe, 2004).

In the context of plant viruses, a robust and specific antiviral RNA silencing response is triggered, and AGOs play a pivotal role in orchestrating this process (Baumberger and Baulcombe 2005). Antiviral AGOs establish connections with small RNAs derived from viruses, enabling them to inhibit viral RNAs or DNAs that possess complementary sequences. Additionally, they also associate with endogenous small RNAs, thereby regulating the expression of host genes and bolstering the defense against viral infections (Carbonell and Carrington 2015).

The number of AGO proteins encoded by plants varies across different species. For instance, Arabidopsis has 10 AGO proteins (Vaucheret, 2008), chilli possesses 12 (Lei Qin *et al.*, 2018), tomatoes have 15 (Bai *et al.*, 2013), maize have 18 (Qian *et al.*, 2011), and rice harbors 19 (Kapoor *et al.*, 2008). The 12 AGOs of chilli were phylogenetically grouped into four different clades based on their resemblance to tomato AGO proteins. Group I consisted of four AGO proteins (AGO1a, AGO1b, AGO10a, AGO10b), group II and III included AGO5, AGO2 and AGO7 while group IV

comprised AGO15, AGO6, AGO4a, AGO4b, and AGO4d. Among the different AGOs, AGO1a, AGO1b, AGO2, AGO4a, AGO10b were significantly over expressed in response to different RNA plant viruses such as PVY, TMV and CMV (Lei Qin *et al.*, 2018). Based on our current knowledge, it is believed that AGO1 plays a central role and main Argonaut protein involved in first line of defense in response to biotic stresses in plants (Voinnet, 2009; Varallyay *et al.*, 2010). Multiple lines of evidence further support the involvement of AGO1 in antiviral responses. The expression level of AGO1a, AGO1b, AGO4a, AGO4b and AGO5were reported to be significantly higher in tomato plants in response to abiotic stress as well as tomato yellow leaf curl virus infection (Bai et al., 2013). In another study, Gan *et al.* (2017) reported that the expression level of AGO1, AGO6 and AGO7 were significantly increased in flower, stem and root tissue when exposed to abiotic stresses. In rice, the AGO2 protein was reported to enhance the susceptibility to rice black streak dwarf virus (Wang *et al.,* 2021). Further, suppressor proteins of gene silencing, such as Tombusvirus P19, appear to decrease AGO1 levels in infected tissue by enhancing the expression of miR168, which regulates AGO1 through a feedback mechanism (Varallyay *et al*., 2010). Other suppressors facilitate the targeted degradation of AGO1 protein (Csorba *et al.,* 2010) or inhibit its RNA cleavage activity, as demonstrated in Cucumber mosaic virus (CMV) 2b (Zhang *et al.,* 2006) and Turnip crinkle virus (TCV) P38 (Azevedo *et al*., 2010). Ahypomorphic *Arabidopsis* mutant of ago1 was found to be more susceptible to virus infections compared to wild-type plants (Morel *et al.,* 2002). Collectively, these findings emphasize the significance of AGO1 protein as the major player involved in antiviral defense mechanisms in plants.

While multiple studies have emphasized the crucial involvement of AGO1 in the host plant's response to various RNA viruses and abiotic stress, there is currently a lack of research regarding its expression levels in response to DNA viruses. Given that DNA viruses can induce both transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) in the host plant, it becomes imperative to investigate the expression levels of AGO1 in response to such DNA viruses. Considering the above background, this study was conducted to test the differential expression levels of AGO1a and AGO1b in response to chilli leaf curl virus infection, a DNA virus under the genus *Begomovirus,* infecting chilli.

MATERIALS AND METHODS

A. Raising of chilli seedling and their transplantation

Chilli seeds of the Pusa Jwala variety were sown in a plastic pot measuring 7 × 6 cm. The pots were filled with a soil mixture consisting of coco pit, vermiculite, and perlite in a ratio of 4:2:1, respectively. The pots were placed in trays containing NPK solution and placed in a glasshouse. After one week of germination, seedlings were transplanted to plastic pots of the same size filled with the same soil mixture. The trays containing the pots were then kept in a glasshouse with a temperature maintained at 30 ± 3 °C and a light and dark period of 14/10 h.

Maintenance of whitefly culture and virus inoculation

B. Maintenance of healthy whitefly culture

Aviruliferous whiteflies were cultivated on either cucumber or brinjal plants and the uncontaminated population were maintained in an insect-proof growth chamber. The growth chamber was set at a temperature of 26 ± 2 °C and followed a light and dark cycle of 16/8 h.

C. Acquisition access feeding and inoculation feeding of ChiLCV

Using an aspirator, aviruliferous whiteflies were collected from the insect house and introduced into a plastic cage containing ChiLCV-infected chilli plants. The cage was then placed in darkness for a period of 24 h to allow the healthy whiteflies to feed on the infected plants, thereby acquiring the virus from the phloem tissue. The following day, the virulent whiteflies were collected from the cages using the aspirator and released onto test plants kept inside the cages. A minimum of 30 viruliferous whiteflies were released per plant. These plants were kept in the cages for a duration of 48 h, after which the whiteflies were eliminated by spraying 0.1% Imidacloprid insecticide. Uninoculated plants were treated as controls.

D. Sample collection, RNA extraction, cDNA preparation

Samples were pooled into three replicates from virus inoculated and uninoculated plants and were collected at 10 days post virus inoculation (dpi) and 25 dpi. Total RNA was extracted using the triazole reagent (G-biosciences, USA). Firstly, 100 mg of leaf tissue was ground into a powdered form using a sterilized mortar and pestle along with liquid nitrogen. Subsequently, 1 mL of triazole reagent was added and thoroughly mixed. After 1 min, 200 µl of chloroform was introduced to the tube, which was then vigorously shaken for 15 seconds. This was followed by centrifugation at 12,000 g for 15 min. Carefully, 400 µl of the resulting supernatant was transferred to fresh microcentrifuge tubes, and 500 µl of ice-cold isopropanol was added. The mixture was incubated at room temperature for 15 min to precipitate the RNA. The precipitated RNA was then pelleted through centrifugation at 12,000 g for 10 min, and the supernatant was discarded. The RNA pellet underwent two washes with 70% ethanol and was subsequently air-dried. Finally, the RNA pellet was dissolved in 40 µl of nuclease-free water. The concentration of the extracted RNA was determined using a Nabi UV/Vis Spectrophotometer (µ2 MicroDigital, Korea) and stored at -80 °C for future use. To synthesize complementary DNA (cDNA), the Revert Aid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) was utilized. A PCR tube with a final volume of 12 µl was prepared, consisting of 2 µL of RNA template (1 µg), 1 µl of oligodT primer (100 nM), and the remaining volume filled with nuclease-free water. The tube was incubated at 65 °C for 5 min in a thermocycler and then

promptly transferred to ice. Next, 8 µL of the cDNA reaction mixture was added to the tube, comprising 4 µL of 5X buffer, 2 µL of dNTP mix, 1 µL of reverse transcriptase, and 1 µL of RNase inhibitor enzyme. The tube was given a quick spin to mix the components. The cDNA synthesis was carried out by incubating the tube in a thermocycler at 42 °C for 1 h, followed by a step at 75 °C for 10 min.

E. Gradient PCR of AGO1a, AGO1b and Ubiquitin primer

Before performing semi-qPCR, melting temperatures (Tm) of the design primers pairs specific to AGO1a and AGO1b and Ubiquitin (an internal control) gene of chilli were optimized through gradient PCR by setting the temperature ranges between 54 °C to 64 °C. Details of the primers were given in the table 1. PCR was conducted using a 25 µl reaction mixture containing 2.5 µl of 10X buffer, 0.5 µl of 10 mM dNTP, 0.5 µl each of forward and reverse primers, 0.125 µl of *Taq* polymerase, 1 µl cDNA and 19.875 µl of nuclease-free water. After a short spin of 3 sec, the tube was placed in a thermocycler with an initial denaturing temperature of 98˚C for 3 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 54 °C to 64 °C for 30 sec and extension at 72 °C for 30 sec. A final extension step was given at 72˚C for 10 min. The PCR product was load into 1.2 % agarose gel and run for 30 min at 85 mV.

F. Semi-qPCR and qPCR

Semi-qPCR was performed to check the increase in the expression of AGO1 in ChiLCV infected plants as compared with the uninoculated control plants. PCR was conducted in a 25 µl reaction volume as mentioned above, using AGO1a (BM-997F/BM-998R) and AGO1b (BM-999F/BM-1000R) specific primer pairs and 100 ng of cDNA template. Further, to quantify and compare the relative level of AGO1 expression, qPCR was performed in 10 µl reaction volume containing 5 µl of 2X SYBR green mix, 3 µl of nuclease-free water, 0.5 µl each of the forward and reverse primer, BM-1021F/BM-1022R (AGO1a) and BM-1023F/BM-1024R (AGO1b), and 1 µl of the template DNA (100 ng). In semi-qPCR and qPCR, the primer pair, BM-1283F/BM-1284R that targets the ubiquitin gene of chilli was used as internal control.

RESULTS AND DISCUSSION

A. Optimization of annealing temperatures

Through gradient PCR, it was observed that the primer pairs specific to Ubiquitin gene (BM-1283F/BM-1284) that was used as internal control were able to give good amplification in all the temperature ranges (Fig. 1a). So, the Tm was kept at 60°C and followed the same temperature in further experiments. Further, the optimum annealing temperature for AGO1a (BM-997F/BM-998R) and AGO1b (BM-999F/BM-1000R) specific primer pairs were kept at 62°C and 58°C, respectively (Fig. 1b, 1c). Following the same gradient PCR, other two primer pairs, BM-1021F/BM-1022R (AGO1a specific) and BM-1023F/BM-1024R (AGO1b specific), used for performing qPCR were kept at 60 °C.

B. Differential expression of Argonaut transcripts in response to ChiLCV infection

The relative expression of two Argonaut proteins, AGO1a & AGO1b, a central protein involved in RNA silencing mechanism, primarily responsible for the first line of antiviral defense in plants were estimated through semi-qPCR and qPCR analysis. Gel electrophoresis of the PCR amplified product showed the presence of specific bands at 505 bp (AGO1a specific), which suggested the over expression of the AGO1a mRNA in response to ChiLCV at 25 dpi as compared to the control, where no amplification was observed in the gel at 30 cycles of PCR (Fig. 2a). In contrast, no amplification of AGO1b mRNA was observed in both the virus inoculated and non-inoculated plants. This might be possible due to the presence of AGO1b mRNA at a low level in both the control and the virus infected plants. To overcome this limitation and obtain a better quantification of the expression level of AGOs mRNA, qPCR was performed. It was observed that both the AGOs (AGO1a & AGO1b) were found to be significantly increased in its expression level after the virus infection. The expression level of AGO1a transcripts was increased up to 1.58 folds at 10 dpi and 4.12 folds at 25 dpi in comparison with the expression level of non-inoculated healthy plants (Fig. 3a). Likewise, the AGO1b mRNA was also observed to be over expressed in response to ChiLCV infection at both 10 dpi (1.75 fold increase) and 25 dpi (1.93 fold increase) (Fig. 3b). The increased expression level of the two AGOs can be correlated with the increase in available Argonaut proteins in the host, which will direct the plant to enhance its gene silencing mechanism and to thereby restrict the invading virus. The increase expression level of AGO1 mRNA in response to virus infection was shown previously in different plant-virus interactions (Azevedo *et al.,* 2010; Várallyay *et al.,* 2010; Wang *et al*., 2011; Zhang *et al.,* 2006). But it is interesting to note that the increase in the mRNA level of AGO1 does not always correlate with an increase in the level of AGO1 protein in the host plant. This is due to the fact that the invading viruses also try to counteract the RNA silencing mechanism in the plants by producing VSRs. The Tombusvirus p19 VSR protein was reported to induces a specific miRNA (miR168) that binds to AGO1 mRNA and leads to the reduction in its translation into proteins (Várallyay *et al.,* 2010). So, the induction of AGO1 in response to virus infection is a part of the host defense response whereas, the parallel induction of miR168 in the host could be considered as a part of viral counter measures against the host defense. Both the responses were found to occur hand-in-hand during the evolution of host pathogen interaction and this knowledge can be exploited in the development of a strategy for plant disease management approaches.

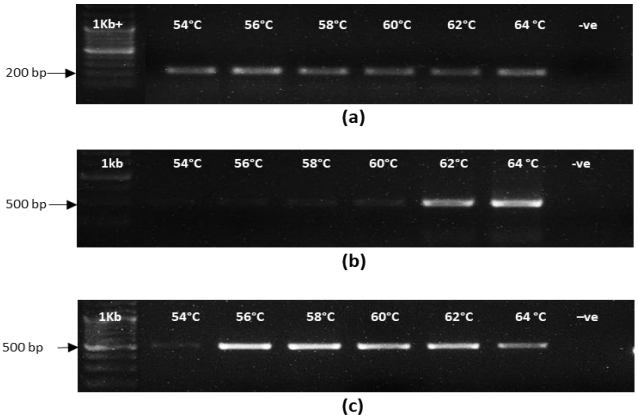

Fig. 1. Gradient PCR of different primer pairs, (a) Ubiquitin control primer (b) AGO1a primer and (c) AGO1b primer, for the optimization of annealing temperatures (Template used for PCR: cDNA from chilli plants infected with ChiLCV).

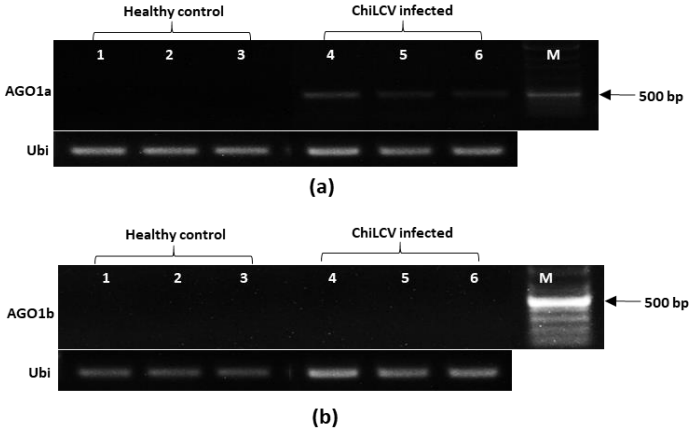

Fig. 2. Semi-qPCR of (a) AGO1a & (b) AGO1b genes in healthy and virus infected chilli plants at 25 dpi. Ubiquitin (Ubi) gene was used as internal control. Lane 1, 2 & 3: Healthy control; lane 4, 5 & 6: ChiLCV infected plants. PCR cycle performed: 30X.

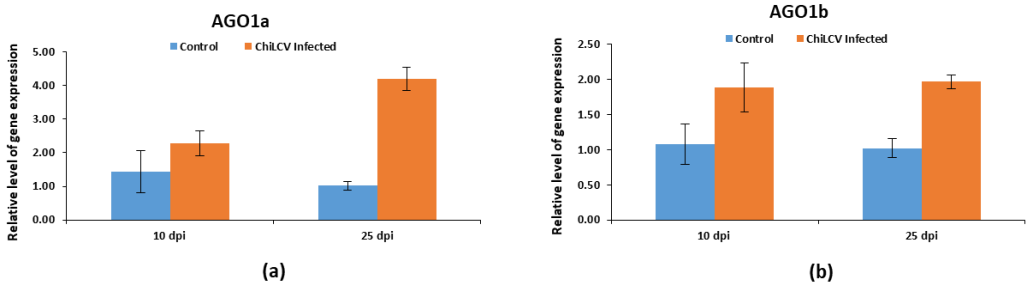

Fig. 3. qPCR assay to check the differential expression of (a) AGO1a and (b) AGO1b transcripts level in healthy control and virus infected chilli plants. Ubiquitin gene was used as internal control for normalization.

Table 1: Details of the primers used in the study.

Primer detail	Primer code	Sequences	T_m (°C)	Amplicon size (bp)
AGO1a (semi qPCR)	BM-997F	AGGTGGAGGT(C/T)ACTCATCGTG	62	506
	BM-998R	TATTCCA(C/T)TGGCCCACTTGTG		
AGO1b (semi qPCR)	BM-999F	GCT(G/T)CA(A/G)GTTCTGGATATT GT	56	551
	BM-1000R	ACAATCTTGCAGACTTCCATTG		
AGO1a (qPCR)	BM-1021F	GAGAGCTGGCGTGG(T/C)TTCTA	60	132
	BM-1022R	CCGATTCAGAAGCTGGCTCA		
AGO1b (qPCR)	BM-1023F	AGAGGTGTGAAGGTGGAGGT	60	196
	BM-1024R	CAACTTGCAGACAAGGCCAC		
Ubiquitin (Ubi)	BM-1283F	GCCAAATACGAGACCACTGC	60	196
	BM-1284R	AAGACCCGTTCCTTGACAACC		

CONCLUSIONS

In this study, the expression level AGO1 gene, a central regulatory protein involved in inducing the first line of defense in plants against viruses was investigated in response to ChiLVC infection. Semi-qPCR and qPCR analyses revealed significant upregulation of AGO1a and AGO1b transcripts in virus-infected plants compared to control plants. The over expression of AGO1a and AGO1b suggests their crucial roles in the defense response against invading viruses. This research contributes to our understanding of the involvement of AGO1 proteins in plant antiviral defense mechanisms in chilli against chilli leaf curl virus infection.

FUTURE SCOPE

The future research can explore the involvement of other AGO proteins in the plant's defense against DNA viruses. This could provide a comprehensive understanding of the role of AGO proteins in antiviral responses. Also, the role of miRNAs in regulating the translation of Argonaut mRNAs into proteins in response to virus infection needs to be understood. Proper understanding of the interaction between AGOs and its corresponding miRNAs and the regulation of its expression under the influence of virus can potentially lead to the development of novel strategies for engineering viral resistance in plants. By manipulating the expression levels or activity of these AGO proteins and miRNAs, it may be possible to enhance plant defense against viruses and improve crop productivity.

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Conflict of Interest. None.

REFERENCES

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M. A., Lagrange, T. and Voinnet, O. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes & Development*, *24*(9), 904–915.

Bai, M., Yang, G. S., Chen, W. T., Mao, Z. C., Kang, H. X., Chen, G. H., Yang, Y. H. and Xie, B. Y. (2012). Genome-wide identification of Dicer-like, Argonaute and RNA-dependent RNA polymerase gene families and their expression analyses in response to viral infection and abiotic stresses in *Solanum lycopersicum*. *Gene*, *501*(1), 52–62.

Baulcombe, D. (2004). RNA silencing in plants. *Nature*, *431*(7006), 356–363.

Baumberger, N. and Baulcombe, D. C. (2005). *Arabidopsis* ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proceedings of the National Academy of Sciences*, *102*(33), 11928–11933.

Carbonell, A. and Carrington, J. C. (2015). Antiviral roles of plant ARGONAUTES. *Current Opinion in Plant Biology*, *27*, 111–117.

Csorba, T., Lózsa, R., Hutvágner, G. and Burgyán, J. (2010). Polerovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. *The Plant Journal*, *62*(3), 463–472.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, *391*(6669), 806–811.

Gan, D., Zhan, M., Yang, F., Zhang, Q., Hu, K., Xu, W. and Liang, D. (2017). Expression analysis of argonaute, Dicer-like, and RNA-dependent RNA polymerase genes in cucumber (*Cucumis sativus* L.) in response to abiotic stress. *Journal of Genetics*, *96*, 235-249

Kapoor, M., Arora, R., Lama, T., Nijhawan, A., Khurana, J. P., Tyagi, A. K. and Kapoor, S. (2008). Genome-wide identification, organization and phylogenetic analysis of Dicer-like, Argonaute and RNA-dependent RNA Polymerase gene families and their expression analysis during reproductive development and stress in rice. *BMC Genomics*, *9*(1), 451.

Morel, J. B., Godon, C., Mourrain, P., Béclin, C., Boutet, S., Feuerbach, F., Proux, F. and Vaucheret, H. (2002). Fertile Hypomorphic *ARGONAUTE*(*ago1*) Mutants Impaired in Post-Transcriptional Gene Silencing and Virus Resistance. *The Plant Cell*, *14*(3), 629–639.

Qian, Y., Cheng, Y., Cheng, X., Jiang, H., Zhu, S. and Cheng, B. (2011). Identification and characterization of Dicer-like, Argonaute and RNA-dependent RNA polymerase gene families in maize. *Plant Cell Reports*, *30*(7), 1347–1363.

Qin, L., Mo, N., Muhammad, T. and Liang, Y. (2018). Genome-Wide Analysis of DCL, AGO, and RDR Gene Families in Pepper (*Capsicum annuum* L.). *International Journal of Molecular Sciences*, *19*(4), 1038.

Várallyay, É., Válóczi, A., Ágyi, Á., Burgyán, J. and Havelda, Z. (2010). Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. *The EMBO Journal*, *29*(20), 3507–3519.

Vaucheret, H. (2008). Plant ARGONAUTES. *Trends in Plant Science*, *13*(7), 350–358.

Voinnet, O. (2009). Origin, Biogenesis, and Activity of Plant MicroRNAs. *Cell*, *136*(4), 669–687.

Wang, X. B., Jovel, J., Udomporn, P., Wang, Y., Wu, Q., Li, W. X., Gasciolli, V., Vaucheret, H. and Ding, S. W. (2011). The 21-Nucleotide, but Not 22-Nucleotide, Viral Secondary Small Interfering RNAs Direct Potent Antiviral Defense by Two Cooperative Argonautes in *Arabidopsis thaliana*. *The Plant Cell*, *23*(4), 1625–1638.

Wang, Z., Chen, D., Sun, F., Guo, W., Wang, W., Li, X. and Zhou, T. (2021). ARGONAUTE 2 increases rice susceptibility to rice black streaked dwarf virus infection by epigenetically regulating HEXOKINASE 1 expression. *Molecular Plant Pathology*, *22*(9), 1029-1040.

Zhang, X., Yuan, Y. R., Pei, Y., Lin, S. S., Tuschl, T., Patel, D. J. and Chua, N. H. (2006). *Cucumber mosaic virus* -encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes & Development*, *20*(23), 3255–3268.

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