Summary & Critique of “Understanding the Cas13 dependent and independent silencing in plants” presented by Divya Mishra.

Introduction

The rise of diverse plant viral infections has prompted the invention and development of numerous genome engineering approaches. Although CRISPR/Cas9 has been in use for a while, this DNA editing technique that uses double-stranded breaks has the potential to cause DNA changes that could be harmful to the species that have been treated. Wrongful mutations can cause unforeseen and unwanted outcomes, particularly in genes with pleiotropic effects (Gratten & Visscher 2016). Because RNA is involved in many physiological activities and can be specifically spatiotemporally controlled, unlike DNA, it has been suggested that manipulating RNA is a safer and more effective option (Reardon 2020). Current RNA degradation technologies related to RNA interference (RNAi) struggle with considerable off-target silencing. The more recently discovered CRISPR/Cas13 system, which targets single-stranded RNA (ssRNA), overcomes these limitations in plants. Therefore, this method presents innovative, more effective ways to accurately control plant features and fight off viruses.

Summary

Although the Cas13 system has been extensively researched in mammalian animals, few writers have been able to draw on any systematic research into this system's functions in plants (Cox et al. 2017). Mishra’s research synthesized the coding sequence for two Cas13a proteins called LbuCas13a (*Leptotrichia buccalis*) and LbaCas13a (*Lachnospiraceae bacterium*) for expression in plants in order to evaluate the Cas13 system. By focusing on the plant-infecting Turnip Mosaic Virus (TuMV), the *in planta* function of these proteins was examined. The TuMV

genome was modified to express GFP, enabling visualization of the Cas13 activity by fluorescence analysis. Mishra differentiates between different types of guides. A single-guide and multi-guide each include an antisense sequence to respectively one or three specific regions of the TuMV genome. In the empty-guide, only the direct repeat (DR) sequence was present.

Nicotiana benthamiana plant leaves were agroinfiltrated to assess the effectiveness of the different systems. Only the procedure including the Cas13 protein and the crRNA guide was expected to result in a decrease in GFP, indicating that TuMV was effectively being silenced. It was anticipated that a Cas13-free treatment wouldn't silence any cells. The same holds true for Cas13 proteins that have an empty or non-targeting guide (NT). The observed outcome differed from the predictions as reduced GFP expression was detected in both systems containing the crRNA guides. This implied that TuMV silencing occurred in both a Cas13 dependent and independent way. Thus, crRNA guides alone, in the absence of Cas13, can elicit viral target RNA reduction. Mishra attempted to systematically review all the relevant literature. Notwithstanding, she found no pre-existing research addressing these findings.

The research investigated endogenous target RNA to ascertain whether this observation was brought about by the fact that it was viral RNA. Unlike host-derived RNAs, viruses have distinct characteristics and can alter the physiology of the host (Jaafar & Kieft 2019). Virus-induced gene silencing (VIGS) down-regulates specific mRNAs, and plays a crucial role in plants' defense mechanisms (Dommes et al. 2019). Mishra's experiments targeted endogenous phytoene desaturase (*PDS*) mRNA using single- and multi-guide crRNA, both with and without Cas13. The bottom leaves of N. *benthamiana* were agroinfiltrated through Tobacco Rattle Virus

(TBV). The NT-guide, the antisense *PDS*, and the empty-guide system were employed as vector controls, negative controls, and positive controls, respectively. A reduction of mRNA levels was observed in both the Cas13 dependent and independent systems, revealing that the crRNA guide alone can affect both viral and endogenous RNA. The term "guide-induced gene silencing" refers to the Cas13-independent system (GIGS). The findings from these studies suggest that a possible area of future research would be to investigate GIGS as an alternative to previously described RNA interference and silencing techniques in plants. However, due to its recent discovery, GIGS has only been reported in a single paper, covering Mishra's research (Sharma et al. 2022).

The second issue presented in this research was how precisely GIGS functions. The presence of short antisense sequences in both crRNA and small RNA (sRNA) used in the endogenous RNAi system, implied that GIGS could function through the same mechanism (Wilson & Doudna 2013). In an attempt to explore this hypothesis, sRNA generation for multiple guides was analyzed using sRNA-sequencing. A single, sharp peak was produced by the single-guide systems. Three unique peaks and a large amount of sRNA were produced by multi-guide devices that targeted three different areas. Despite some sRNA being independent of the targeted transcript, the majority of this sRNA matches the targeted sequence. Additional evaluation of the produced sRNA revealed that the majority of them were 21 nucleotides long. These findings were viewed as a significant indicator that GIGS works through endogenous components of RNAi, since sRNA, specifically with a length of 21 nucleotides, is the distinguishing feature of RNAi (Elbashir et al. 2001).

The research engages with current knowledge about RNAi to ascertain whether this process is involved in the GIGS mechanism. Experiments designed to specifically disrupt the vital parts of the RNAi system were carried out to investigate this hypothesis. Important information about the proteins involved in the silencing mechanism was disclosed by base-pair mismatch mutagenesis. In eukaryotes, RNAi is performed by members of the Argonaute (AGO) protein family which use the sequence data provided in the sRNA as a guide to determine which complementary RNAs should be targeted for silencing. In order to effectively silence a gene, AGO cleaves the RNA backbone between the crucial nucleotides 10 and 11 (Fang & Qi 2016, Sheu-Gruttadauria & MacRae 2017). In the mutagenesis experiments, there was some RNA decrease as a result of base pair mismatches between 5 and 6 or 21 and 22. A full inhibition of RNA interference was brought on by base pair mismatches in 10 and 11. This is an indication that GIGS might function through RNAi and AGO.

To put this work into a broader context, it was crucial to determine if this GIGS was solely active in *N. benthamiana*, or whether other plants used a similar technique. Mishra's study used photobleaching studies on the plant *Solanum lycopersicum* and came to the conclusion that GIGS and Cas13 are functional in every plant species. The heritability of these systems was examined in *Arabidopsis* using the pleiotropic regulatory gene *TRANSPARENT TESTA GLABRA1 (TTG1)*. This study examined anthocyanin biosynthesis across generations, demonstrating heritable phenotypes for numerous variables in stable transgenic *Arabidopsis* that were mediated by both Cas13 and GIGS. The Cas13 dependent RNA targeting has been reported in, among others, *Oryza sativa*, *Ipomoea batatas*, and *Glycine max* (Kavuri et al. 2022). Although initial observations suggest that GIGS may be expected in these plants, among others,

these findings should not be extrapolated to all species unless specific experiments are carried out to verify this in each species.

This study further presented intriguing guide-parameters that could be applied to improve GIGS. First, a minimum crRNA length of 22 nt is needed. Shorter guides are ineffective. Second, increasing the number of crRNA in a multi-guide led to stronger photobleaching. However, the expression of various single guides and their combination into a multi-guide could result in large levels of sRNA production and off-target silencing. Although some sRNA may be produced by repeatedly expressing the same single guide into one guide, there would overall be very little off-target effect. However, this account must be approached with some caution because the rules governing the interactions between sRNA and target mRNA are not fully understood yet (Karunanithi et al. 2020).

Critique

Mishra spent a considerable amount of time on explaining the traditional CRISPR-Cas system and the variants that have been investigated in studies and experiments over the past few years. She outlined the similarities and differences between Cas9 and Cas13. She also discussed additional mechanisms or techniques for editing RNA, illuminating how proteins like Dicer and AGO play a part in RNA interference and silencing. Emphasizing that RNAi causes greater off-target effects compared to other systems, she presented her two research questions, revealing the goal or topic for this seminar. Is Cas13 better than RNAi in plants? Can we develop Cas13-dependent gene knockout in plants for modulating gene expression?

Mishra developed a method for determining the Cas13-dependent silencing against TuMV in plants. Both orally and visually, this approach was introduced and described to the

audience. She also articulated and supported the expected results of her experiment, encouraging the audience to consider the science behind the information being provided. The predicted and experimental outcomes were presented in an understandable way, giving sufficient consideration to previous research. In her experiments, Mishra further explores the ways in which RNA reduction could be induced by crRNA guides alone in the absence of Cas13. The translation of viral RNA may be constrained by a regulated decrease in translational capability as a result of the defensive mechanisms being engaged by the identification of virus-associated molecules or RNA, indicating that viral RNA itself can cause RNA reduction (Jaafar & Kieft 2019). Difficulties arise when an attempt is made to study RNA silencing with viruses. VIGS is a common plant RNAi-mediated defense mechanism, making it challenging to distinguish between reactions against viral RNA and host RNA (Velasquez 2009). It is important to bear in mind the possible bias in the results.

In an attempt to study whether endogenous RNA also triggered GIGS, Mishra designed an experiment in which plant leaves were exposed to four different target guides. She highlighted and explained three controls that should be used. The NT-guide, the antisense PDS, and the empty guide served as vector controls, non-target controls, and positive controls, respectively. Once more, the findings were visually presented in a clear, comprehensible, and thoughtful way, comparing and contrasting the various treatments with the controls. Mishra acknowledges the significance of the finding of GIGS. However, no attempt was made yet to quantify the association and distinction between GIGS and VIGS. The paper also suggests that GIGS might function through RNAi, but it appears to be ambitious in its claims. The research

would have been more significant if a wider range of knockout mutations had been performed for proteins involved in RNAi, such as Dicer and AGO.

When probing new subjects, such as specific elements of RNAi, Mishra reiterated the procedure to ensure the audience understood why she had chosen to include this aspect of her research as well as how precisely her original research questions had changed and new ones had emerged during the course of the experiments. To elucidate her reasoning and make an effort to connect with the audience, she maneuvered strong illustrative slides.

Mishra, however, did not always exude the energy and body language necessary to captivate the audience when presenting. Adopting a louder, slower pace would have been beneficial. Additionally, nonverbal communication could have been improved. The findings presented in the presentation would have left a bigger impact on the audience if Mishra's body language supported and advocated them. Mark Bowden, a body language consultant, contends that specific nonverbal communication is needed to influence and persuade others around how trustworthy and credible your message is (Bowden 2010).

Graduate and undergraduate students with a wide range of prior knowledge were present in the audience for Mishra's lecture, in addition to career researchers with Ph.D.s and other degrees. Notwithstanding, the data and study were presented in a way that was understandable for diverse academic levels, particularly when techniques like CRISPR and RNAi were introduced and repeatedly explained. Mishra was able to respond with clarity and precision to queries on methodology and perspective, both simple and complex questions. Mishra was accommodating when students tried to actively participate in the discussion by

asking her questions. She appeared more comfortable in this smaller discussion group, as seen by her body language.

Conclusion

Plant engineering and virus treatment now have intriguing new options thanks to Divya Mishra's discovery of Cas13-dependent and independent RNA silencing. I was ecstatic to learn about this novel process as a genetics student who has a keen interest in the use of biotechnology in animals, plants, and food. I am hoping that we will be able to unravel the exact nature of this system, and manage to use this to our plants' advantage. The presentation's slides and organizational structure were excellent and encouraged audience participation and active thinking. The audience may have been more affected overall if the presentation's use of verbal and nonverbal cues had been stronger and more convincing. This lecture piqued my interest in plant virology and agricultural applications as well as the potential implications of this finding for plant biology as a whole.

Work Cited:

Cox, D., Gootenberg, J., Abudayyeh, O., Franklin, B., Kellner, M., Joung, J., Zhang, F., (2017). RNA editing with CRISPR-Cas13. Science, 358(6366), 1019-1027.

Dommes, A., Gross, T., Herbert, D., Kivivirta, K., Becker, A., (2019). Virus-induced gene silencing: empowering genetics in non-model organisms. Journal of Experimental Botany, 70(3), 757-770.

Elbashir, S., Lendeckel, W., Tuschl, T., (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes and Development, 15(2).

Fang, X., and Qi, Y., (2016). RNAi in Plants: An Argonaute-Centered View. The Plant Cell, 28(2), 272-285.

Gratten J., and Visscher, P., (2016). Genetic pleiotropy in complex traits and diseases: implications for genomic medicine. Genome Medicine, 8(1), 78.

Jaafar, Z., and Kieft, J., (2019). Viral RNA structure-based strategies to manipulate translation. Nature Reviews Microbiology, 17(2), 110-123.

Karunanithi, S., Oruganti, V., de Wijn, R., Drews, F., Cheaib, M., Nordström, K., Simon, M., Schulz, M.H., (2020). Feeding exogenous dsRNA interferes with endogenous sRNA accumulation in *Paramecium*. DNA Research, 27(1).

Kavuri, N., Ramasamy, M., Qi, Y., Mandadi, K., (2022). Applications of CRISPR/Cas13-Based RNA Editing in Plants. Cells, 11(17).

Reardon, S., (2020). Step aside CRISPR, RNA editing is taking off. Nature, 578(7793), 24-27.

Sharma, V.K., Marla, S., Zheng, W., Mishra, D., Huang, J., Zhang, W., Morris, G.P., Cook, D.E., (2022). CRISPR guides induce gene silencing in plants in the absence of Cas. Genome Biology, 23 (1), 6.

Sheu-Gruttadauria, J., and MacRae, I., (2017). Structural Foundations of RNA Silencing by Argonaute. Journal of Molecular Biology, 429(17), 2619-2639.

Velasquez, A., Chakravarthy, S., Martin, G., (2009) . Virus-induced Gene Silencing (VIGS) in *Nicotiana benthamiana* and Tomato. Journal of Visualized Experiments, 28.

Wilson, R., and Doudna, J., (2013). RMolecular Mechanisms of RNA Interference. Annual Review of Biophysics, 42(1), 217-239.