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Title

Identification of Cis-Elements Responsible for D14-LIKE2 Transcription

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Author

Mendez, Jose

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Dr.
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Dr. Richard Cardullo, Howard H Hays Jr. Chair, University Honors

Abstract

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Acknowledgements

INTRODUCTION

Karrikins are butenolide compounds that were discovered in smoke residue following wildfires and were found to promote seed germination in a variety of plant species such as Grand Rapids lettuce (Flematti et al. 2004). Over the years, the effects karrikins have on other plants have been uncovered, and its effects can vary in several species. For example, in tomato, okra, maize and rice, karrikin treatment leads to increased shoot and root growth (Kulkarni et al. 2006; van Staden et al. 2006). When *Arabidopsis thaliana* is treated with karrikin, its seed and seedlings become more sensitive to light, while also allowing karrikins to act as positive regulators for light-dependent development (Waters MT 2017). Also, in *A. thaliana,* a pathway was discovered involving karrikin and the receptor protein known as KAI2 (KARRIKIN INSENSITIVE 2). It was discovered that when KAI2 perceives karrikin, KAI2 then interacts with the SCF (Skp, Cullin, F-box containing) complex, leading to the degradation of a protein known as SMAX1 (SUPPRESSOR OF MAX2 1) (Figure 1A) (Nelson et al. 2015).

Karrikins are known to drive expression of several genes in plants such as *Arabidopsis thaliana*, one of these genes being *DLK2* (DWARF14-LIKE 2). Through testing and measuring the transcript levels of *KAI2, AtD14 (Arabidopsis* DWARF14 orthologue), and *DLK2* in response to GR24 (which is a molecule analogous to karrikin) and using water as control, it was discovered that *DLK2* is highly upregulated in response to karrikin treatment (Figure 1C). In testing, *DLK2* transcripts were found to show a large increase in abundance, in comparison to *AtD14* and *KAI2* which barely responded to GR24 treatment. In past experiments, when the function of *DLK2* is disrupted, there is no observable change in phenotype when compared to wildtype *A. thaliana* (Végh, Attila, et al. 2017). However, we are still interested in *DLK2* due to it being so highly upregulated by karrikin treatment since this makes it a useful transcriptional marker for KAI2

activity. Our goal is to identify the cis-elements, or regions of non-coding DNA, that regulate the

transcription of karrikin responsive genes like DLK2 in order to unravel how plants perceive

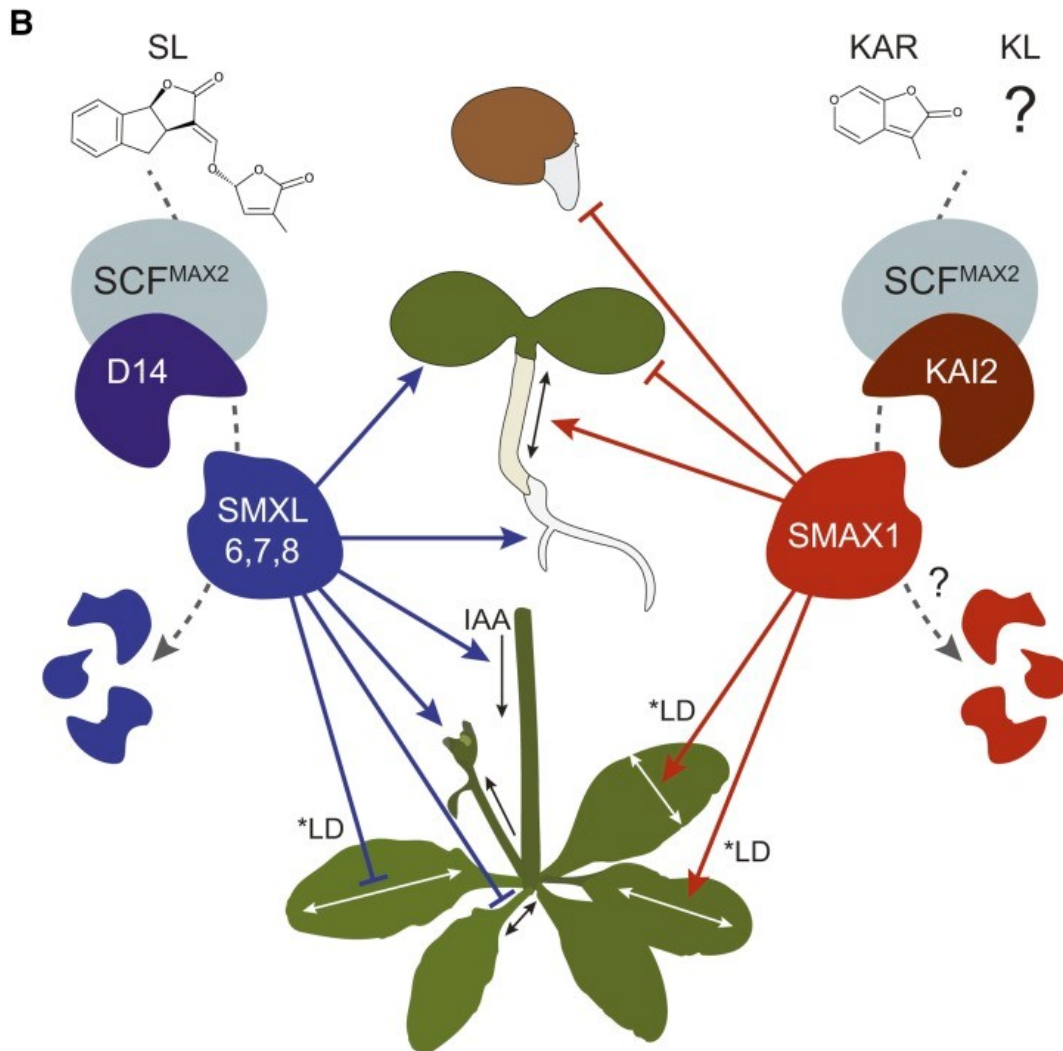karrikin, and to discover what is happening at the molecular level during perception of karrikin.

Figure 1A: Complex model of Strigolactone (SL) and Karrikin signaling pathway. D14

(DWARF 14) and KAI2 are both receptor proteins which mediate karrikin and SL perception.

DLK2 is thought to act in a similar way to *D14* and *KAI2* in that it somehow perceives karrikin

and initiates a transcriptional response, however, it lacks interaction with *MAX2* (Nelson et. al 2015).
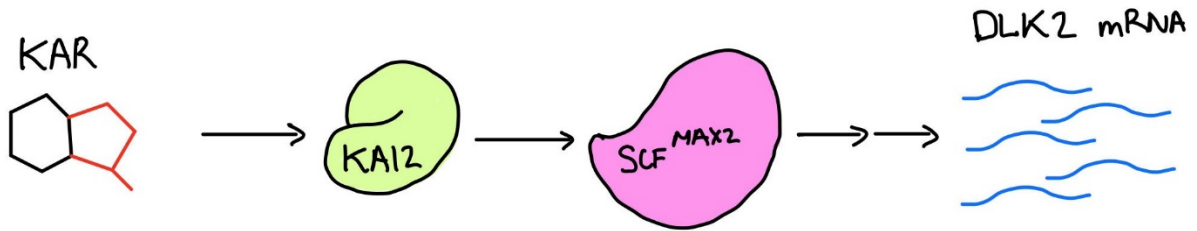

Figure 1B: Simple model depicting when KAI2 perceives karrikin, *DLK2* expression is increased, however, exactly which other proteins are involved hasn't been determined yet.
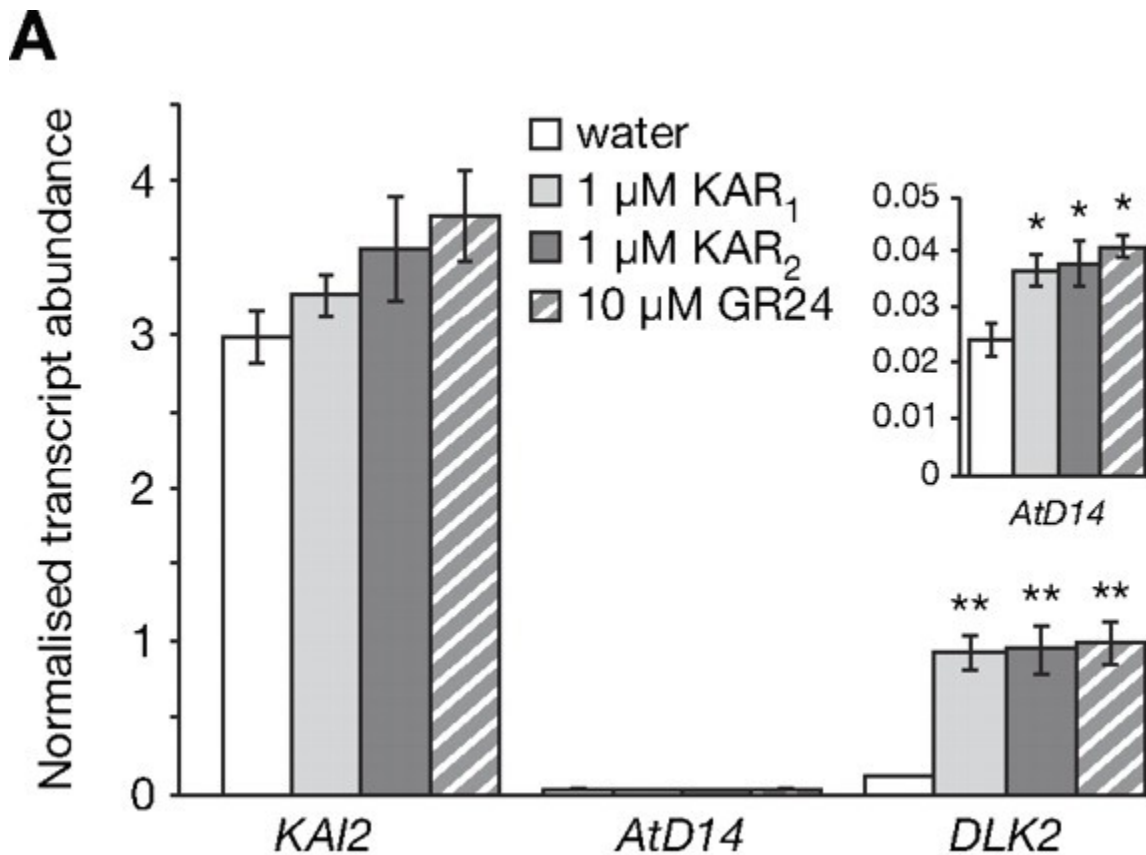
Figure 1C: Data demonstrating a minimal response from *KAI2 and AtD14* to karrikins or GR24, while *DLK2* transcripts increase in abundance several fold (Waters MT et al. 2012).

Cis-regulatory elements are regions of non-coding DNA, which contain binding sites for transcriptional factors that activate transcription of genes. My research project relies on data produced from two transcription factor binding site discovery assays, Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) and DNA Affinity purification sequencing (DAP-seq). ATAC-seq inserts sequencing adapters into regions of open chromatin, using a hyperactive Tn5 transposase, which allows for generation of a map of regions of potential transcription factor (TF) binding, DAP-seq instead uses affinity-purified transcription factors, coupled with next-generation sequencing of a genomic DNA library, to create another map of potential TF binding sites.

To identify potential cis-elements in the DLK2 promoter, we examined recently published DAP-seq and ATAC-seq data sets (O'Malley et al. 2016 & Maher et al. 2017). We found two regions of interest in the region upstream of DLK2, and another region of interest in the DLK2 intron, based on peaks of potential transcription factor binding sites identified from the published DAP-seq data. To determine whether these regions mediate karrikin responsiveness, we are are using CRISPR-CAS9 to remove these regions of interest, to see how they affect karrikin activation of DLK2 transcription.
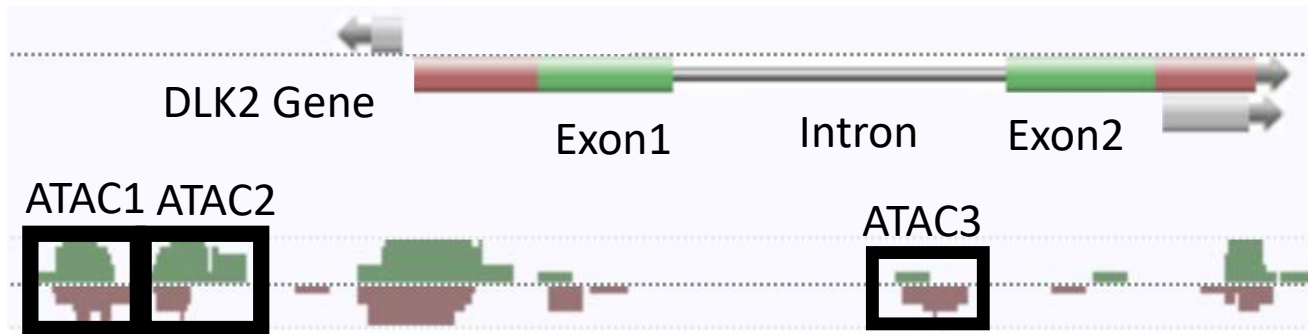
Figure 2: Data from previous DAP-seq and ATAC-seq studies were utilized to find three regions of interest within the DLK2 promoter region, the region upstream of the actual DLK2 gene (O'Malley et. al 2016). Regions of interest were found by looking for points where the green/brown histograms showed significant peaks. The regions highlighted in black, ATAC1 (-1100 to -870 bp), ATAC2 (-790 to -490 bp), and ATAC3 (1380 to 1580 bp), were the regions that would be targeted for CRISPR-CAS9 deletion. It is interesting to note that one of the peaks is located within the intron of the DLK2 gene.

Another well studied plant hormone is auxin, for which researchers were able to find a transcription factor binding site motif that was targeted by auxin treatment. Using this discovery, they created a highly active synthetic auxin response element (AuxRE), which they called DR5, by combining repeats of this motif to form a synthetic promoter (Murfett, J, et al. 1997). DR5 has been used extensively in investigating responses to auxin and has proved to be valuable in advancing the research in that field. Assuming the transcription factor binding sites can be narrowed down the same way for DLK2, the main goal would be to create a DR5-like synthetic promoter, that could then be used as a reporter gene for karrikin response.
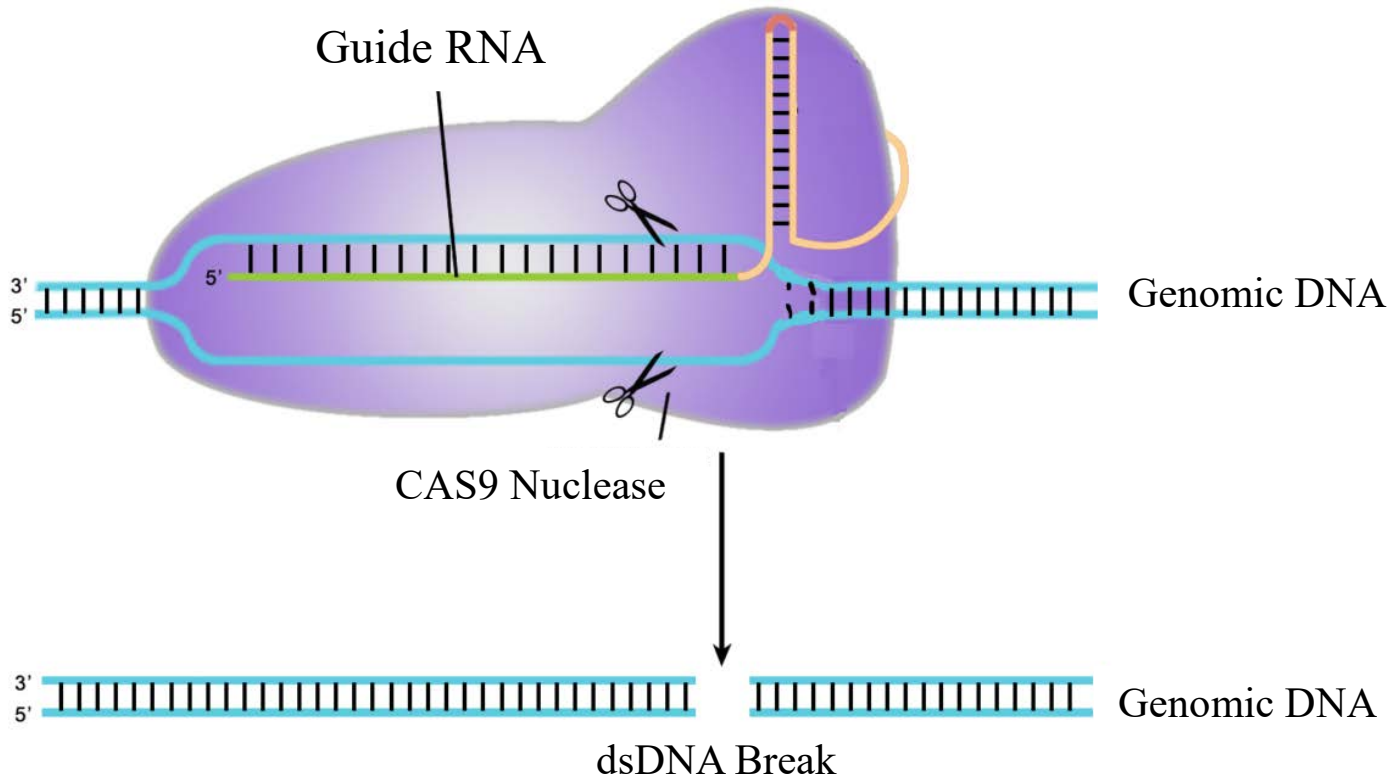
MATERIALS AND METHODS

CRISPR-CAS9

Figure 3: Model of the CRISPR-CAS9 system. Guide RNA's can be designed to be recognized by the CAS9 Nuclease enzyme create a double-stranded cut at a specific site of DNA (Hamer 2017).

In order to best investigate if these regions, found from DAP-seq and ATAC-seq data, play a role in the perception of karrikin, CRISPR-CAS9 mediated deletion was used to remove two regions of the DLK2 promoter, the ATAC1 and ATAC2 regions. The plan was to also remove the region of ATAC3 in the DLK promoter, but unfortunately this was not able to be accomplished and will be completed later. These large deletions of the promoter were done by using pairs of guide-

RNA's to direct the CAS9 enzyme to make two separate double-stranded cuts, flanking each of the ATAC1 and ATAC2 regions.

	Guide RNA Primer Sequences
AtDLK2_ATAC1-DT1-BsF	ATATATGGTCTCGATTGTGAGTGAAGTTAAGGCCAAGTT
AtDLK2_ATAC1-DT1-F0	TGTGAGTGAAGTTAAGGCCAAGTTTTAGAGCTAGAAATAGC
AtDLK2_ATAC1-DT2-R0	AACTCATATGTATAGCATAACTTCAATCTCTTAGTCGACTCTAC
AtDLK2_ATAC1-DT2-BsR	ATTATTGGTCTCGAAACTCATATGTATAGCATAACTTCAA
AtDLK2_ATAC2-DT1-BsF	ATATATGGTCTCGATTGTAAATACCAGTTTCCCCGGTGTT
AtDLK2_ATAC2-DT1-F0	TGTAAATACCAGTTTCCCCGGTGTTTTAGAGCTAGAAATAGC
AtDLK2_ATAC2-DT2-R0	AACTGCTAATCATAGGAGTCATCAATCTCTTAGTCGACTCTAC
AtDLK2_ATAC2-DT2-BsR	ATTATTGGTCTCGAAACTGCTAATCATAGGAGTCATCAA

Table 1: Primers listed above were the guide RNA's used to create the CRISPR-CAS9 deletions.

To do this, two separate PCR reactions were performed on the ATAC1 and ATAC2 regions of the DLK2 promoter, using primers designed specifically to contain what are known as PAM sites – the sites at which the CAS9 nuclease enzyme can recognize and cut double-stranded DNA.

Once the PCR products of the ATAC1 and ATAC2 sites had been isolated, both products were then put through a Goldengate reaction, which basically ligated the products within a destination vector known as pHEE401E (Wang et al., 2015) – which is the vector that contains the genetic information for the CAS9 nuclease enzyme.

	Detection Primer Sequences
DLK2_ATAC1-F	CGGTATGGTGTGGATCTGTGAC
DLK2_ATAC1-R	GTAAAACCAGAGATATCTGGGCG
DLK2_ATAC2-F	CCAAGTCATCTCACGTGTACTG
DLK2_ATAC2-R	GTCAATGAAGAATAAAGCACACGGC

Table 2: Primer sequences listed above were used to detect whether deletions were made after transforming the CRISPR-CAS9 constructs into *A. thaliana.*

Bacterial Transformation via Electroporation

Bacterial transformation of *E. coli* electrocompetent cells, DH5a, were used in order to take advantage of the cells machinery to create copies of our DNA of interest. Electroporation is a process by which electrocompetent cells are shocked, briefly opening the pores of the cell membrane, allowing for the introduction of foreign DNA. After transformation of DNA into electrocompetent cells, colonies of bacteria are selected on antibiotic plates, in order to confirm that our DNA of interest was inserted into the cell. The DNA of interest is carried by a vector which contains a sequence for antibiotic resistance, so only colonies carrying this DNA vector will grow on the antibiotic media. Further confirmation is performed via PCR, using gene-specific primers which will only amplify DNA if the DNA is present. Once the DNA sequence of interest is isolated from *E. coli* colonies, the plasmid is then purified and sent out for Sanger Sequencing to check for any errors in the DNA sequence that might've arose during PCR.

Arabidopsis thaliana Plant Transformation

Plant transformation was performed in order to investigate if the deletions of the ATAC1 and ATAC2 regions would have any phenotypical changes in vivo in *Arabidopsis thaliana.* Transformation was done by using the pHEE401E vector, including with the region of interest, as a destination vector to be transformed into electrocompetent agrobacterium cells, GV3101. Once the agrobacterium cells were transformed, selected on antibiotic plates, and confirmed via PCR, the cells were then cultured and used to transfect *A. thaliana* plants in order to introduce the CRISPR constructs into *A. thaliana.* Once plants have been transfected, the seeds that are produced are then plated on antibiotic selection media. The pHEE401E vector contains an antibiotic resistance gene, Hygromycin, which allows for this selection. Seeds which are able to grow healthy on the antibiotic plates are chosen to be grown into the next generation.

Arabidopsis thaliana Plant Genotyping

An important step in creating the DLK2 promoter mutants is to make sure that the deletion is homozygous, meaning that the deletion is present on both chromosomes. This is done by segregating through multiple generations, in order to find mutants that are homozygous for the deletion. The deletions themselves are confirmed by using primers which flank the regions where the deletion was supposed to occur. On an agarose gel, there are three potential bands that will come as a result from PCR. If there is no deletion, a band of the normal size of the promoter will result. If there is a heterozygous deletion, there will be one normal band and another band of reduced size due to the deletion. And lastly, if there is a homozygous deletion then there will just be one product band of reduced size.

While doing this genotyping it is also important to make sure to segregate lines which no longer contain the CAS9 cassette, the cassette containing the genetic information for the CAS9 nuclease enzyme. Having mutant lines which still contain the CAS9 cassette can cause problems in future generations, since it is more likely that the CAS9 can have off target activity and cause more double stranded breaks at undesired locations across the genome, which is why it is very important to get rid of the CAS9 cassette at the earliest generation possible.

Hypocotyl Assay

Hypocotyl assays were performed on the ATAC1 and ATAC2 mutant lines in order to test phenotypic changes in response to karrikin. Seeds were sterilized in a solution of 70% ethanol, with 0.05% (v/v) Tween-20, for 10 minutes, then rinsed again in 70% ethanol before being dried off and stored in microcentrifuge tubes. Seeds were then plated on small petri dishes of 0.5 x Murashige and Skook medium (pH 5.7) and kept in dark at 4 degrees Celsius for three days. After the three days, the petri dishes were then sprayed with 10uM karrikin and acetone (for control) using a spray bottle (10 sprays/plate) and placed into a growth chamber and grown in continuous red light for a total of five days at room temperature. After the five days, the plates were taken out and the hypocotyls were immediately measured using imageJ.
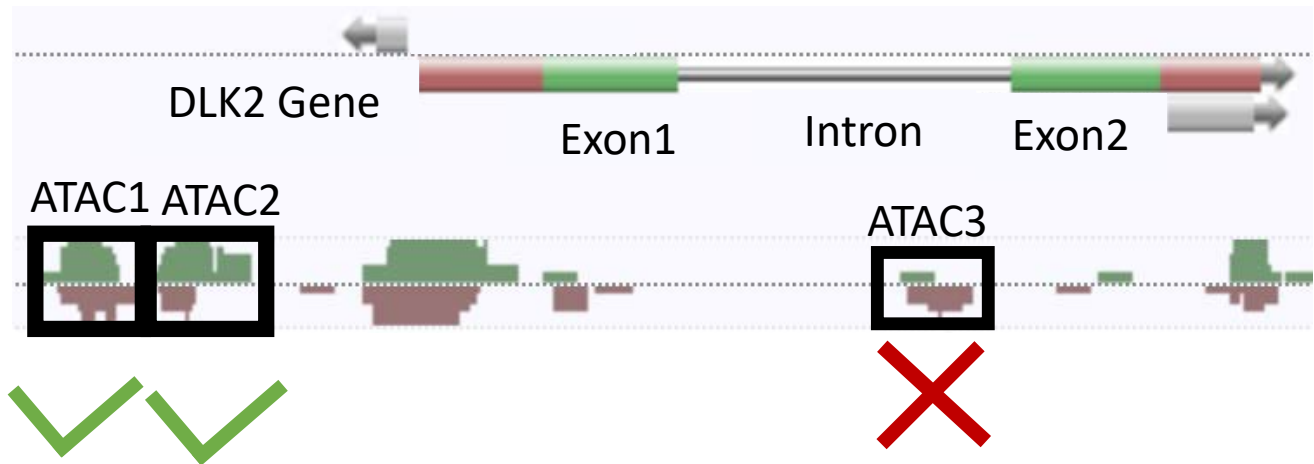
RESULTS

Figure 4: The figure above illustrates which regions of the DLK2 promoter were able to be successfully transformed into *A. thaliana*, those being ATAC1 and ATAC2, while the ATAC3 region lying within the promoter was unsuccessfully transformed.

After identifying three regions of open chromatin upstream of *DLK2*, we decided the best way to investigate whether these regions were important for karrikin perception was to use CRISPR-CAS9 mediated deletion to remove them completely. Transcription factors, or cis-elements, are well known to be important regulators of transcriptional activity, so based on the published DAP-seq data showing potential TF-binding sites, we hypothesized that one or more of these regions could be playing key role in karrikin perception.

Two of the three regions of interest, ATAC1 and ATAC2, were successfully deleted using CRISPR-CAS9. Once the heterozygous deletions were confirmed via PCR (Table 2), the next steps were to obtain a homozygous deletion mutant, which was also lacking the CAS9 cassette in order to avoid any off-target activity/unwanted deletions. Unfortunately, the ATAC3 region located within the intron of the DLK2 gene was not able to be studied, most likely due to faulty

guide-RNA's which weren't successful in making a double-stranded DNA cut at our desired cut sites. It is important to note that while some interesting observations were made, this is very preliminary research data since these experiments were unable to be repeated due to time constraints. Another key point is that although we were successful in creating an ATAC1 homozygous deletion mutant line lacking the CAS9 cassette, the same could not be said for the ATAC2 mutant line. The ATAC2 mutant line did contain a homozygous deletion, but still had the CAS9 cassette present. Nonetheless, we carried on with experiments in order to see if preliminary results could be observed.

After obtaining the two homozygous deletion mutants, hypocotyl assays were performed on the ATAC1 and ATAC2 dlk2 mutant lines. Hypocotyl assay was deemed the best route for investigation, due to its advantages of allowing for the observation of how karrikin would be perceived in our dlk2 mutant lines. A couple other advantages to hypocotyl assay are that it aligns nicely with previous studies done on DLK2 in order to have more comparable data, and it also would've allowed for the extraction of RNA to be used in Real Time PCR.

When studying the ATAC1 mutant line, it was concluded that there was no phenotypical difference of the mutant line compared to wild-type *Columbia-0,* meaning that the hypocotyl lengths between the ATAC1 and wild type were basically the same. This finding was not surprising, considering that in past publications DLK2 mutants haven't shown a phenotypical difference when compared to wild type (Végh et al. 2017).

However, when it came to the ATAC2 dlk2 mutant line, a change in phenotype was observed. The results of the hypocotyl assay demonstrated a significant decrease in hypocotyl length of the dlk2-ΔATAC2 mutant line when compared to wild type *Columbia-0.* Although this finding came as a surprise, concrete conclusions on whether this phenotype change was true have yet to be

made since these experiments haven't been repeated. This decrease in hypocotyl length was

supported further once the hypocotyls were measured and plotted using imageJ and R (Figure

5b). When looking at the violin plot data, there is a clear decrease in hypocotyl length of the

dlk2-ΔATAC2 mutant, when compared to that of wild type.

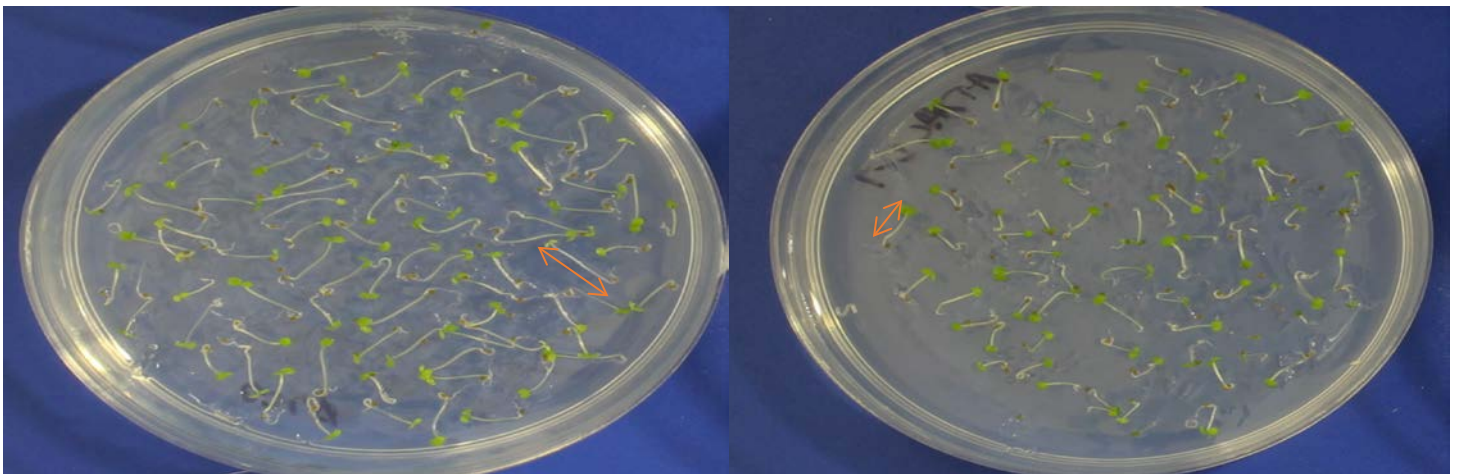WT-COL0   ΔATAC2

Figure 5a: The figure above shows the result of the hypocotyl assay for the dlk2-ΔATAC2 line.

It can clearly be seen that the hypocotyls of the mutant ATAC2 line are significantly shorter than

those of the wild type hypocotyls (orange arrows highlight length difference).
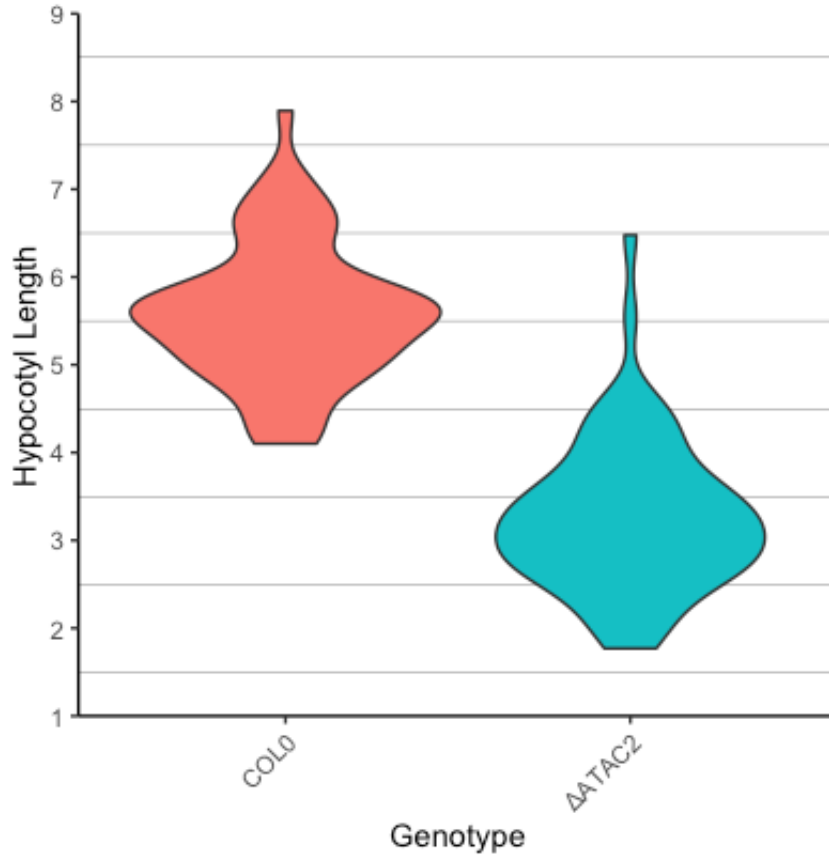
Figure 5b: The violin plot above shows the significant difference between wild type *Columbia-0* and the mutant ATAC2 line. The majority of data points are represented by the widest part of the graph, while the top and bottom of each plot represent the range of the data, as well as the outliers of the dataset. The sample size was 50 hypocotyls measured for each genotype, and the units of measurement was in (mm). Measurements were done on 5-day old seedlings, immediately after growth in continuous red-light.

DISCUSSION

Observing that the hypocotyl lengths of the mutant ATAC2 line were shorter than that of wild type was the most surprising find out of these experiments. Again, this data is purely preliminary data so none of it can be proven for sure until these experiments are actually repeated with mutant lines with the CAS9 cassette absent. However, if they are repeatable, it will change the way that the DLK2 gene has been studied and can lead to a range of different experiments to be done in the future.

Figure 6: The figure above is from a previous hypocotyl assay done with a mutant dlk2 line, showing that there is no phenotypical difference when compared to wild type (Végh et. al 2017).

The main question to be asked is why this change in hypocotyl length is being observed when past hypocotyl assays haven't shown this. In past hypocotyl assays done with loss-of-function dlk2 mutants, no significant change in phenotype was observed, while overexpression mutants showed a slight increase in hypocotyl length (Végh et al. 2017). So, if our phenotype is true, this decreased hypocotyl length would be a very novel finding when compared to past literature. Further investigation is required to answer this, but one hypothesis could be in the way that the mutants themselves were created might be affecting how the mutants in turn affect how the gene is regulated. Past mutants were created using a T-DNA insertion in a different region than in our experiment, while ours took advantage of the CRISPR-CAS9 system to create deletions in the promoter.

A known effect of karrikin treatment is a decrease in hypocotyl length, similar to the length observed in the dlk2-ΔATAC2 mutant line. This observation by itself could hint at a hyperactive KAI2 signaling pathway, like when wild type *A. thaliana* is treated with karrikin (Figure 1A). To test this in the future, we could create a kai2 dlk2-ΔATAC2 double mutant and see if hypocotyl length is restored back to normal in the presence of karrikin. This would tell us whether this phenotype is coming from KAI2 signaling pathway being hyperactive or from something else. It could also be that this dlk2-ΔATAC2 mutation isn't affecting DLK2 expression at all, it could instead be affecting some regulator that dictates where and when DLK2 is expressed in plant tissues, which is leading to this shorter hypocotyl length. Even though *DLK2* shares 42% and 40% amino acid sequence similarity with *KAI2* and *AtD14* (Végh et al. 2017), it differs in the sense that it is unable to bind *MAX2,* which could mean that its function is not that of a signaling protein. Answering this question would require more investigation into what DLK2 is doing at the molecular level. It is known, however, that *D14* can hydrolyze strigolactones while *KAI2*

presumably hydrolyzes KAI2-ligand and karrikin derived signals. Even though it is unknown what substrate *DLK2* might be hydrolyzing, based on the amino acid sequence similarity as well as its response to karrikin, it is known that *DLK2* can in fact act as a hydrolase-based protein similar to *D14 and KAI2* (Végh et al. 2017). What we've hypothesized as a possible explanation, is that instead of *DLK2* acting as a signaling protein, what if it is hydrolyzing a substrate such as KL (KAI2-ligand) and initiating feedback regulation for *KAI2*. If *DLK2* expression is increased perception of karrikin via *KAI2*, this would allow for *DLK2* to regulate the amount of *KL* that is presumably perceived by *KAI2*. However, this explanation is strange considering it would be expected that dlk2 knock-out mutants would have the same effect.

A key experiment that was unable to be completed was to perform Real Time Polymerase Chain Reaction (RT-PCR), which would've allowed for the expression levels of to be compared to wild type DLK2. If the results obtained are true, then this would demonstrate that our CRISPR-CAS9 deletion have had disrupted DLK2 gene function in a way that hasn't been seen before.

Another experiment to be conducted in the future would be to fuse the regions of the promoter that were studied to a luciferase reporter gene and perform transient expression in *Nicotiana benthamiama.* This would allow for further investigation into these specific regions and how/if they influence karrikin perception.

CONCLUSION

In conclusion, it seems that we came across an interesting phenotype in our mutant DLK2 line, ATAC2, which must first be confirmed through repeat experiments. This observation challenges past notions that DLK2 mutants generally do not have a phenotypical change. Once these

experiments are repeated in the future, and if the same observations are made, this can allow for experiments that will eventually lead to a better understanding of how the karrikin regulation occurs.

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