Epigenetic regulation of Gene Associated Transposable Elements in potato:

Effect of DNA methylation in the promoter region of *Wound induced 1*

Internship Report

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Abstract

In Solanacea, transposable elements (TEs) are found in close proximity to genes. Genetic analysis, northern blotting and sequencing revealed that they are the source of numerous small interfering RNAs (siRNAs) which, because of their structure and specific length, are thought to be involved RNA-directed DNA methylation, an epigenetic silencing mechanism. A gene associated transposable element (GATE) located in the promoter of the potato *wound-induced 1* (*Win1*) gene and its influence on the expression of *Win1* are analyzed more closely in the experiments described here. These experiments were designed to test the hypothesis that the siRNAs produced by the GATE guide methylation to complementary DNA sequences in the promoter region, and thus reduce the expression of *Win1*. The methylation status of the *Win1* promoter region was determined in an RNAi line, RNAiRDR2 and in wild type (WT) potato using bisulphite sequencing. The RNA mutant shows lower expression of RNA-Dependent RNA Polymerase 2 (RDR2) and lower levels of siRNAs than the WT line. We were able to show that the reduction in siRNA levels in RNAiRDR2 correlate not only with a 20% reduction in CHH methylation in the *Win1* promoter region, but also with a significant increase in expression of the *Win1* gene

Keywords: Transposable elements, RNA-directed DNA methylation, siRNA, RNAiRDR2 mutant, *Solanum tuberosum*.

Abbreviations

AGO4: Argonaute 4
DCL: Dicer-Like Protein
DdDM: RNA-directed DNA methylation
GATE: Gene-associated transposable element
Pol IV: Nuclear RNA polymerase IV
RDR2: RNA-dependent RNA polymerase 2
siRNA: small interfering RNA
TE: transposable element

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Also, I would like to thank Daniela Pignatta for the nice discussions we had over lunch; Jeffrey Tung for ensuring that everything went always smoothly in the lab; and Colleen Higgins, Pamela Rios and Andrew Lai for their help.

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1. Introduction

The Baker Lab is part of the Plant Gene Expression Center, which is run jointly by the USDA's Agricultural Research Service and the Plant & Microbial Biology Department of the University of California, Berkeley. The lab focuses on the understanding of epigenetic regulatory mechanisms of siRNAs derived from gene-associated transposons (GATEs).

1.1. Transposable elements

Transposable elements (TEs) or transposons are defined as mobile DNA sequences which, through a process called transposition, can change their position in the genome of a single cell (Henderson and Jacobsen 2007; Slotkin and Martienssen 2007). TEs were first described by Barbara McClintock in her genetic studies on maize, and are now known to make up large fractions of eukaryotic genomes (Table 1), with amounts ranging up to 60% in maize (McClintock 1956; Kidwell 2002). Though TEs are known to be major sources of genetic variation, they are often viewed as “parasitic” sequences, and their activity is potentially harmful potential by causing mutations by insertion, deletion or translocation in the host genome (Slotkin and Martienssen 2007). Mechanisms such as histone modification and DNA methylation have thus evolved to down-regulate or silence TE activity. These features cause alterations to the chromatin, leaving TEs intact but in an inactive, "cryptic" state (Henderson and Jacobsen 2007).

Table 1: Amount of transposon derived DNA in percentage of total genome of important species (Kidwell 2002).

Organism	**% of genome derived from transposons**
Yeast - *S. cerevisiae*	3%
Nematode - *C. elegans*	6%
Arabidopsis thaliana	14%
Fruitfly - *D. melanogaster*	15%
Rice - *Oryza sativa*	14%
Homo sapiens	44%
Corn - *Zea mays*	60%

Increasing evidence indicates that transposable elements and mechanisms responsible for their silencing play a major role in the epigenetic regulation of processes within the organism (Li and Baker 2010; Slotkin and Martienssen 2007; Slotkin et al. 2009). Gene Associated Transposable Elements (GATEs) for example, are a special class of transposons, which can act as regulators and influence the expression of certain genes by producing considerable amounts of siRNAs, which in turn can for example guide DNA methylation (Kuang et al. 2009; Li and Baker 2010; Mathieu et al. 2007).

In practical applications, such as breeding for resistance to pathogens, a better understanding of transposable elements and the RNAs they produce and interact with could, for example, help explain how they contribute to certain regulatory features that are invoked by pathogens, or why specific resistance genes from wild relatives may be regulated differently when introduced into domesticated cultivars. Also the sudden appearance of unexpected traits in such crosses could be caused by activation of TEs.

1.2. Epigenetic regulation mechanisms and DNA methylation

Epigenetics is defined as the study of inherited phenotypic changes that are not caused by mutations in the underlying DNA (Henderson and Jacbosen 2007). In this field of science, it is not yet fully understood how genetic sequences are recognized by silencing mechanisms, leading to repression of some and expression of others (Henderson and Jacobsen 2007). These systems are thus of high interest, and siRNAs are thought to play an important role by guiding epigenetic modification mechanisms to specific sequences (Henderson and Jacobsen 2007). Among these effects, cytosine methylation is being intensely studied. It consists in the addition of a methyl group to cytosine bases in different sequence contexts (CG, CHG, CHH, where H is A, T, or C), modifying and regulating the expression patterns of genes (Matzke et al. 2009).

The commonly accepted concept of RNA-directed DNA methylation (RdDM) in the model plant *A. thaliana* assumes that target loci (e.g. a transposon) recruit RNA polymerase IV (PolIV) complexes through a yet unknown process, leading to the production of single-stranded RNA (Henderson and Jacobsen 2007; Law and Jacobsen 2010). Through RNA dependent RNA polymerase 2 (RDR2), double-stranded RNA is made from ssRNA, and will be diced into 24nt-long siRNAs by Dicer-like protein 3 (DCL3). Once loaded into the PAZ- and PIWI-domains of ARGONAUTE 4 (AGO4), the siRNAs will lead this new complex to homologous sequences and guide DRM2, which will methylate the designated DNA sequences

(Law and Jacobsen 2010). At the same time, the production of new siRNAs increases, amplifying the silencing in a loop-like process (Law and Jacobsen 2010). This accumulation is thought to be dependent on RDR2, as mutants with reduced expression of this enzyme show a decrease in siRNAs (Henderson and Jacobsen 2007).

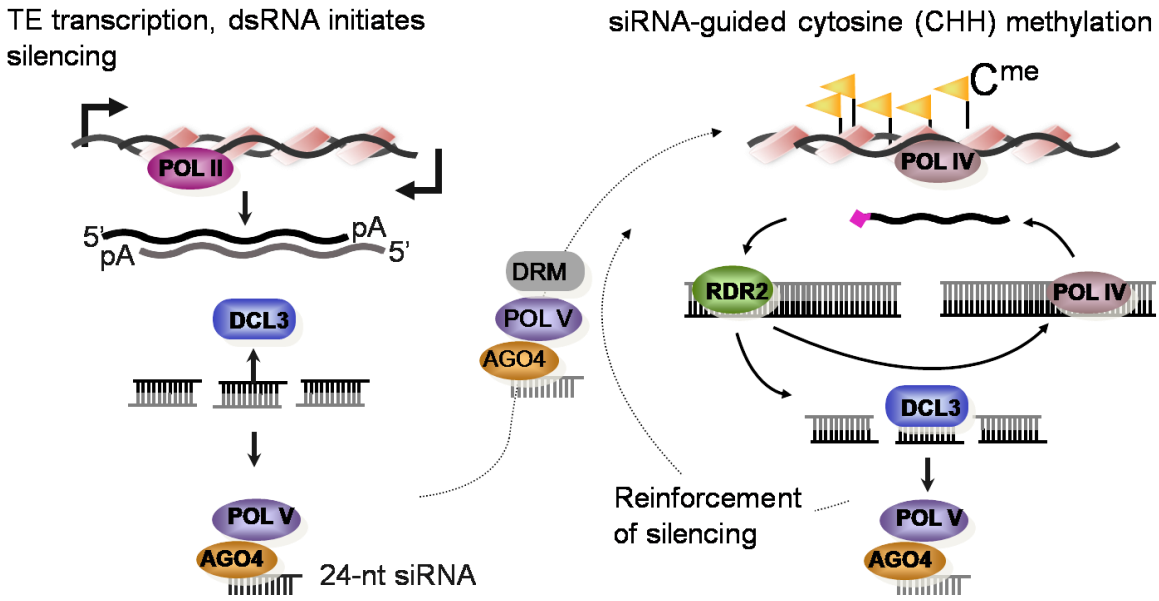

Figure 1: Cycle of RNA-directed DNA methylation (RdDM) in *A.thaliana*. When double-stranded RNA (dsRNA) is formed from RNA transcribed from a transposable element, it is processed by Dicer Like 3 (DCL3) into small fragments of specific lengths. These are taken up by ARGONAUTE4 (AGO4), direct it to complementary DNA sequences and initiate cytosine methylation. An amplification process involving RNA-dependent RNA polymerase then initiates the production of more dsRNA, reinforcing methylation and silencing.

Methylation seems to be particularly important in the regulation of transposable elements, which, in order to have their harmful potential reduced to a level viable for the plant, need powerful control mechanisms (Matzke et al. 2009).

To determine the amount and location of methylation, DNA is treated with bisulfite, which will convert non-methylated cytosines to uracils. Comparing treated and untreated sequences allows for precise determination of the methylation state of the single cytosine residues. This process is illustrated in Figure 2.

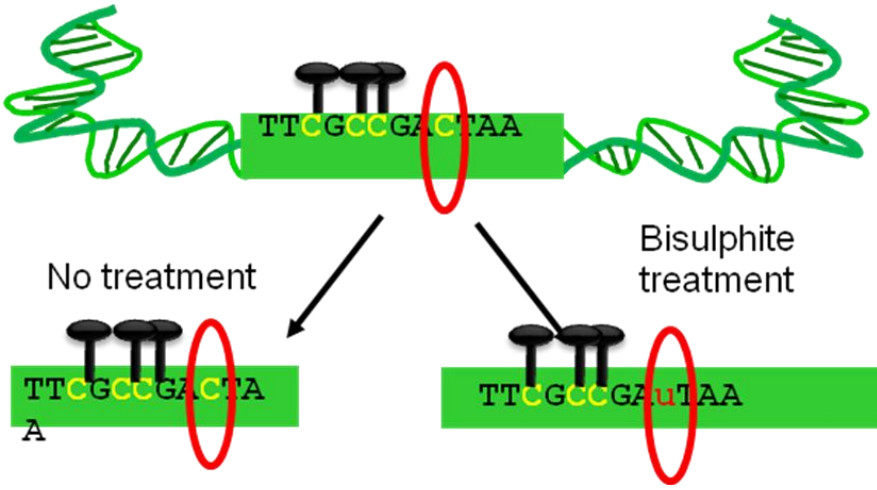

Figure 2: Process of bisulphite sequencing. Treatment with bisulphite converts unmethylated cytosine residues to uracil, while methylated residues remain unaffected. Comparing treated and untreated sequences allows determination of the methylation states of the single cytosine residues.

1.3. Research question

In experiments realized prior to my arrival in the Baker Lab, it was shown that the genomes of Solanacea contain abundant transposons, and that many of them are located in or in close proximity to genes, forming GATEs.

The potato *wound-induced 1 (Win1)* gene was found to contain a MITE (miniature inverted repeat transposable element) named *MITE in Solanacea* 23 (*MiS23)* in its promoter region (149-797bp) (Li and Baker 2010; Oosumi et al. 1995; Stanford et al. 1989). Further investigation through genetic analysis and northern blotting and sequencing showed that the transcription of this TE leads to the production of large amounts of siRNAs of the 24nt size class (Kuang et al. 2009).

These findings lead to the hypothesis that the siRNAs originating from this GATE are involved in the guidance of asymmetric cytosine methylation to complementary target sequences, and can thus have the ability to modulate expression of the nearby *Win1* gene.

This hypothesis was tested by analyzing differences in cytosine methylation in a fragment of the *Win1* promoter (position 150-515) in both a wild type (WT) potato line and the *StRNAi-RDR2* mutant with reduced RDR2 expression. Since this protein is known to be involved in a loop-like process reinforcing DNA methylation by producing more siRNA (see Figure 1), it was expected that the mutant line would show fewer siRNAs, and thus a reduced ability to methylate the *Win1* promoter. This in turn would lead to a higher expression of the gene controlled by this promoter.

Quantification of siRNAs matching the MITE showed that the abundance of siRNAs was in fact clearly lower in the mutant line as can be seen in Figure 3 (B.Baker, personal communication). Comparing sequences from both lines obtained through bisulfite sequencing revealed that this drop in siRNA levels correlates with a 20% reduction in CHH methylation (Figure 4) and an increased expression of *Win1* (Figure 5).

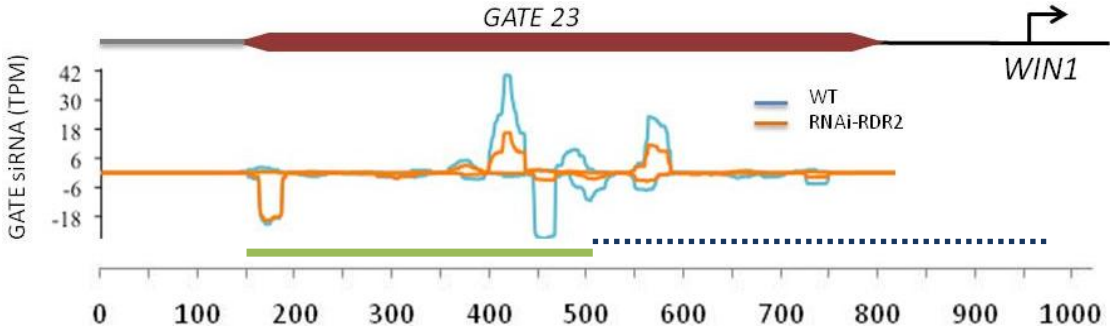

Figure 3: The RNAiRDR2 mutant (orange) shows a clear decrease in siRNA levels compared to the Wild Type line (blue). Unit: Transcripts Per Million (TPM). The siRNAs match precisely to the GATE inserted in the promoter region of the gene. The green solid line represents the portion of the *Win1* promoter region that had previously been sequenced, while as the dotted line indicates the 456bp long region (from bp508 to 964) that was amplified with primers LF1191 and LF1192 and bisulphite sequenced during this project.

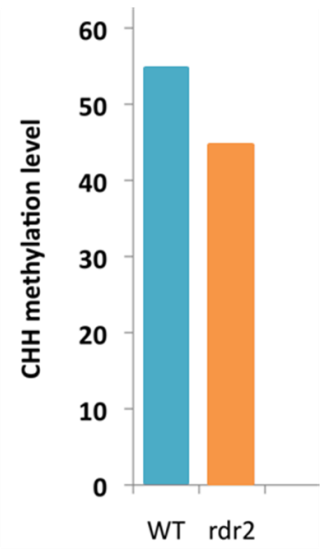

Figure 4: In previous experiments in the Baker lab, bisulphite sequencing of both the *StRNAi-RDR2* mutant and the wild type reveals that CHH methylation is 20% lower in the mutant line, a decrease that correlates with the decrease in siRNA levels (Figure 3).

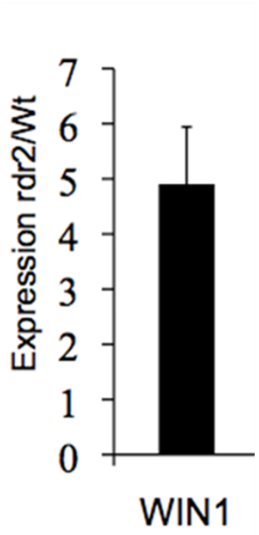

Figure 5: Illustration of the expression of *Win1* in the mutant line in relation to wild type. The mutant shows a clear increase in the gene's expression.

In order to be able to confirm these findings, the remaining part of the *Win1* promoter that had not been sequenced so far was the focus of the present experiment, with the aim of obtaining accurate data on its methylation status. The fragment to be sequenced is shown in Figure 3.

2. Material and Methods

2.2. PCR amplification of desired fragments

In potato, the *Win1* gene is located at position 942-1626bp in the 7kb genomic DNA (GenBank number X13497). Genomic DNA from both WT and StRNAi-RDR2 was treated with bisulfite, and amplified by PCR using primers designed to match specific regions (listed in Appendix 1). The PCR conditions are described in Table 2 and Table 3.

Table 2: PCR reaction "Bisulfite Sequencing"

Reagent	Amount (µl)
DNA template	1
10mM F-primer	0.5
10mM R-primer	0.5
2x Phusion Flash MasterMix	5
ddH_2O	3

Table 3: Conditions for PCR reaction " Bisulfite Sequencing " (30 cylces)

Temperature	Time	
95	5'	
95	15''	30cycles
50	45''	
72	1'30''	
72	6'	

After the first run with the PCR conditions "Bisulfite Sequencing" (Table 2), the same PCR reaction was repeated, using a 50µl reaction volume for the second run. 2µl of both PCR products are then loaded on 1% agarose gel, and run for ~30min at 110V to check for successful amplification.

If this gel showed the bands of interest, 30µl of the PCR products were then run on another gel, and the bands of interest cut out and purified using QIAquick MiniElute (QIAGEN) columns according to the manufacturer's protocol.

2.3. Cloning and colony PCR

The purified PCR product was then cloned into OneShot competent *E.Coli* cells using the Zero Blunt TOPO PCR cloning kit (Invitrogen), according to the manufacturer's protocol.

Table 4: Reaction composition for TOPO cloning

Reagent	Amount (µl)
Fresh PCR product	1
Salt solution	1
PCR 4 BluntTOPO	1
Water for final volume of 5µl	3

The transformed cells were then plated on LB-plates containing kanamycin, and grown overnight at 37°C.

Colonies were picked with a pipette tip and placed into PCR buffer and plated onto numbered plates. The PCR tubes were used to run Colony PCR to check whether the cells contain the constructs of interest, while as the colonies on plates were used for further processing.

Table 5: PCR reaction for Colony PCR.

Reagent	Amount (µl)
5x Buffer Green GoTaq	2
10mM LF799 primer	0.4
10mM LF800 primer	0.4
$MgCl_2$	0.6
ddH_2o	6.2
DNTP	0.2
GoTaq	0.2

Table 6: PCR conditions for program "Feng" used for Colony PCR (34 cycles).

Temp (°C)	Time	
95	5’	
95	15’’	30cycles
55	30’’	
68	2’	
4	forever	

3µl of each reaction were loaded on 1% agarose gels to test for successful transformation. Colonies whose PCR gave the expected bands were incubated in 2ml LB (50µl Kan/50ml LB) overnight on shaker, before being MiniPrepped (QIAGEN) the next day.

2.4. Sequencing

After measuring the DNA concentration of the extracted DNA at 260nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific), sequencing PCR reaction were then run using the following protocol.

Table 7: PCR reaction "Seq".

Reagent	Amount
DNA	~600ng
5x Reaction Buffer	2 µl
BigDye	1 µl
Primer LF799	1 µl
ddH_2O to total of 10 µl	

Table 8: PCR conditions for "Seq", 25 cycles.

Temp (°C)	Time	
96	2’	
96	30’’	30cycles
50	15’’	
60	4’	
4	forever	

The PCR products were then prepared for sequencing using the following protocol as follows: a reagent mix containing 12.5µl ddH2o, 2µl NaAc pH 5.0, and 47.5µl 100% ethanol per reaction was prepared. The PCR products were transferred into 1.5 Eppendorf tubes and 62µl of the reagent mix added to each tube. After 20 minutes of precipitation at room temperature, they were centrifuged at full speed for 20 minutes. The supernatant was removed and the tube washed by adding 500 µl of 70% ethanol. After another 5 minutes of centrifugation at full speed, all ethanol was removed and the tubes let to dry completely. The DNA was then resuspended in 15µl Hi-Di formamide (Applied Biosystems), and loaded into the wells of a sequencing plate suited for an ABI 3130 sequencing machine (Applied Biosystems).

2.5. Data analysis

The raw data from the sequencer is then assembled in the VectorNTI program (Applied Biosystems, USA), and the quality of the data assessed. High quality sequences are then aligned to the original template sequence obtained from NCBI (www.ncbi.nlm.nih.gov*)* in order to find differences in methylation. A program custom written by Dr. Feng Li helped with this step, and produced graphical representation of the data.

2.6. Controls

In order to ensure that the methods were applied correctly, a fragment from an exon of an *S.tuberosum* beta-tubulin sequence that had previously successfully been specified was also analyzed. The 301bp long fragment (GenBank Nr. EU935742, position 3-304) is known to have a very low methylation level, and no differences between WT and RNAiRDR2 are expected. This makes it an optimal sequence to test bisulphite sequencing, which, if successful, should show very low methylation. The fragment was amplified with the primer pair LF981 and LF982.

3. Results

3.1. PCR amplification of promoter fragments

The products of the initial PCR reactions were separated on agarose gels, and if present, the bands of interest extracted for further processing.

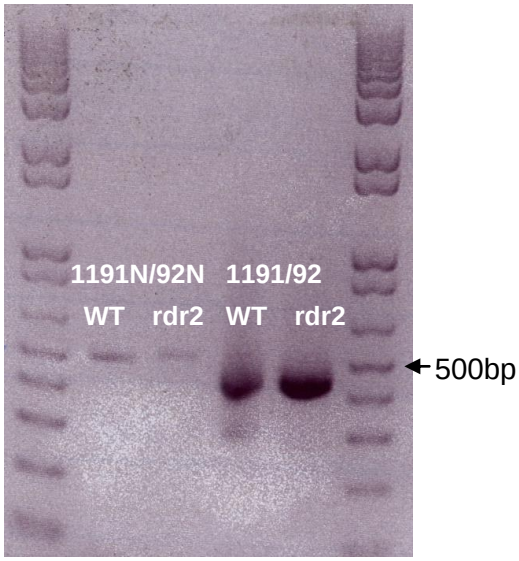

Figure 6: 1% agarose gel showing the product of a "nested" PCR. In the first round (lane 1 and 2), the primer pair LF1191N and LF1192N gave a fragment of 499bp in both WT and RNAiRDR2. These bands were then extracted, and used as a template for a second round, this time using primers LF1191 and LF1192, located inside the previous fragment, and amplifying a new product of 456bp (lanes 1 and 2 for WT and RNAiRDR2, respectively). Size marker: 1Kb+ DNA ladder.

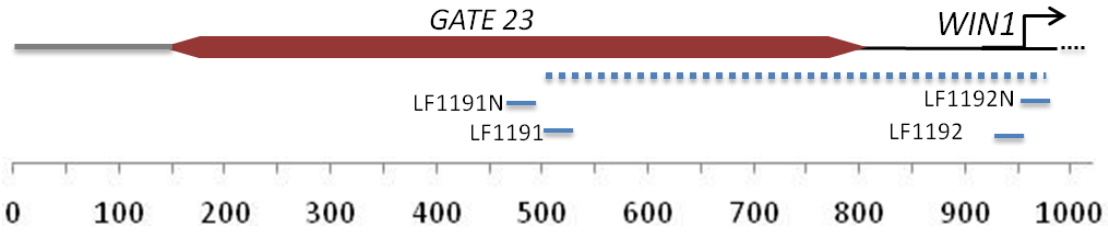

Figure 7: Graphical representation of the Win1 promoter region. The blue dotted line indicates the fragment that was amplified in a "nested" PCR, where the primer pair LF1191N and LF1192N were used in a first round, succeeded by a second round of PCR with primers LF1191 and LF1192 for higher specificity.

3.2. Cloning and colony PCR

The bands on agarose gels representing the desired fragments were extracted, purified and then transformed into competent *E.coli* cells using the Zero Blunt TOPO PCR cloning kit (Invitrogen). Transformed cells were then selected for on kanamycin containing LB plates. Colony PCR was used to ensure that the selected colonies in fact contained the fragments of interest.

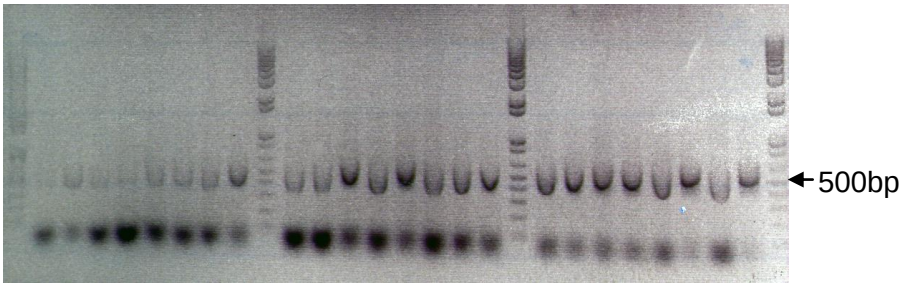

Figure 8: 1% agarose gel showing bands obtained from 24 colonies transformed with a fragment amplified from the RNAiRDR2 mutant with LF1191/92. Once the presence of the desired fragment proven, colonies containing it were put into culture so as to obtain enough DNA for sequencing. Size marker: 1Kb+ DNA ladder.

3.3. Sequencing & data analysis

The sequences obtained through Sanger sequencing were aligned and the methylation status graphically represented as shown in **Error! Reference source not found.**. Over the whole sequence, the wild type showed lower CHH methylation than the mutant line: 78.88% versus 82.83%. If only the sequence corresponding to the *MiS23* insertion was considered, WT showed slightly higher methylation: 92.6% versus 90.8%.

Table 9: Comparison of methylation status between WT and RNAiRDR2 as seen over the whole sequenced fragment.

	RNAiRDR2		WT	
C-context	**Number of Cs**	**%Methylated**	**Number of Cs**	**%Methylated**
CG	4.92	98.6	5	88.5
CHG	2	46.5	2	21.4
CHH	70	82.83	70	78.88

Table 10: Comparison of methylation status between WT and RNAiRDR2 limited to the sequence corresponding to the *MiS23* insertion.

	RNAiRDR2		WT	
C-context	**Number of Cs**	**%Methylated**	**Number of Cs**	**%Methylated**
CG	3	100	6	100
CHG	0	0	0	0
CHH	47	90.83	47	92.61

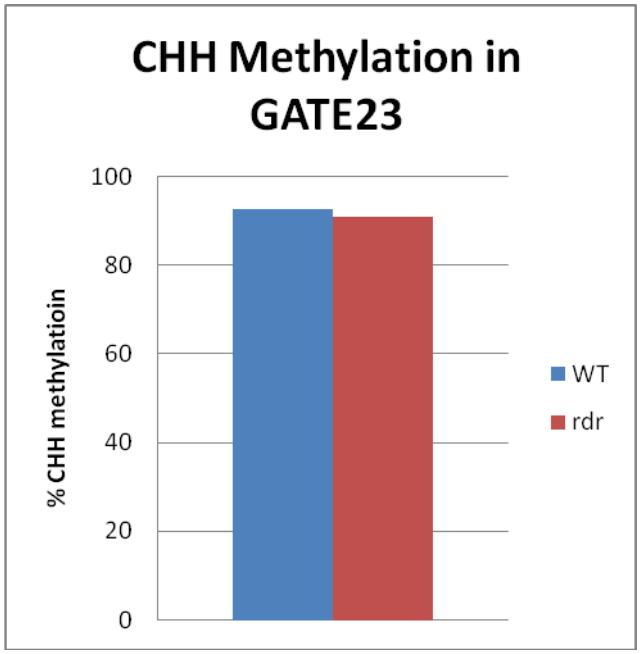

Figure 9: CHH methylation in the part of the sequence corresponding to the MiS23 insertion (508-797bp). Tthe Mutant line shows only slightly reduced methylation.

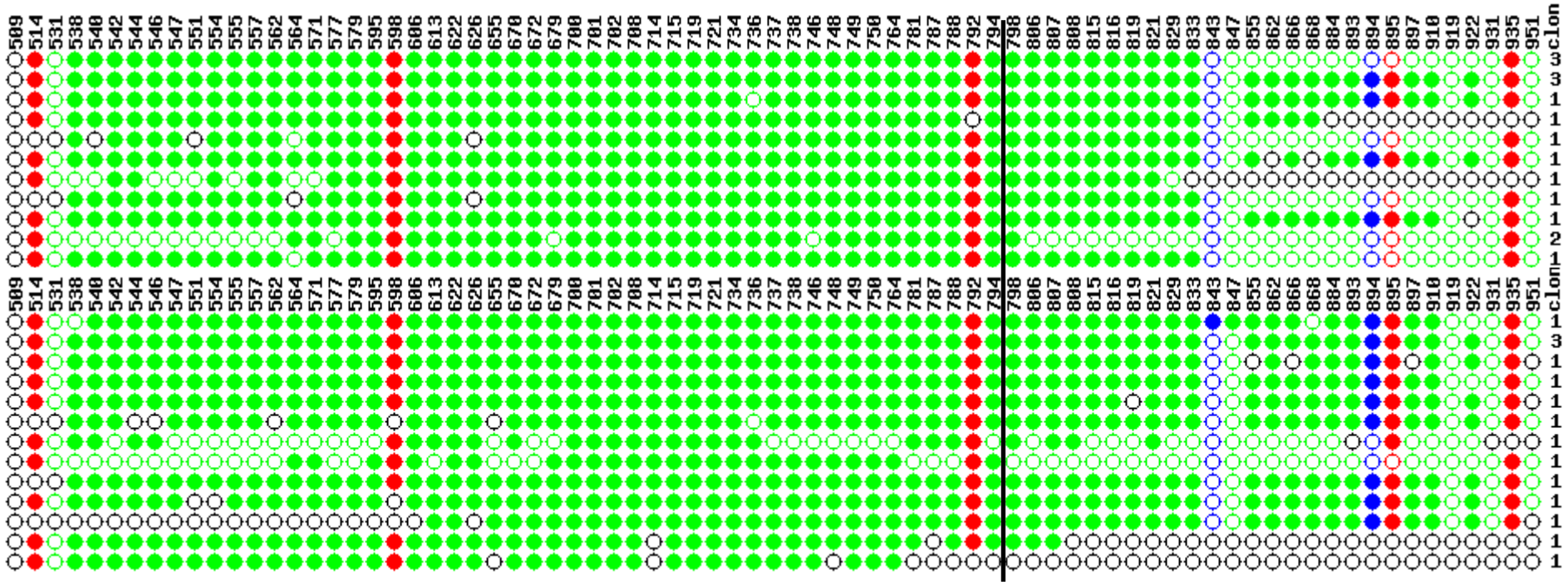

Figure 10: Graphical representation of the methylation state in the 456bp long fragment in the promoter region of *Win1* (position 508-964), amplified with the primer pair LF1191 and LF1192. Every line represents a methylation pattern, and the number on the right of the line expresses the number of clones that exhibited it. The columns represent each cytosine residue found. The color and fill of the circles expresses the methylation state of a particular cytosine residue, as explained in the legend below. The vertical line at position 797 indicates the end of the *MiS23* (508-797) insertion. While apparent differences in cytosine methylation can be observed between the wild type (Top) and the RNAiRDR2 mutant, it must be kept in mind that some lines have to be multiplied by the number on the right, if several clones showed the same pattern.

Legend

- CHH methyl.
- CHH unmethyl.
- CHG methyl.
- CHG unmethyl.
- CG methyl.
- CG unmethyl
- Not determined

3.4. Controls

Bisulphite sequencing of a 301bp long fragment from an *S.tuberosum* tubulin exon showed, as expected, very low cytosine methylation levels in both WT and RNAiRDR2 (results displayed in Figure 11). This confirmed that the DNA used throughout these experiments had successfully been treated with bisulphite, and that a very high proportion of unmethylated cytosine residues had been converted to uracil.

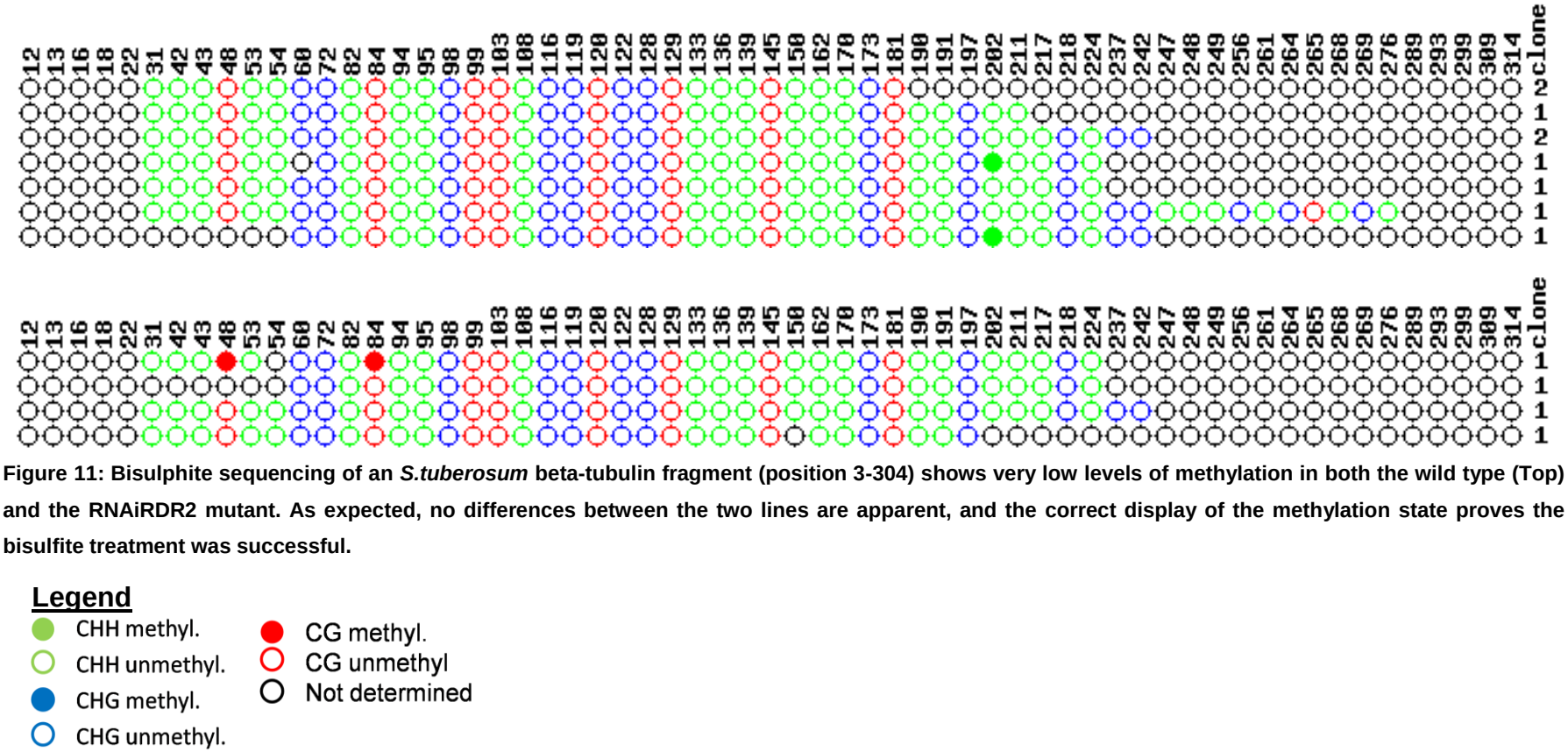

Figure 11: Bisulphite sequencing of an *S.tuberosum* beta-tubulin fragment (position 3-304) shows very low levels of methylation in both the wild type (Top) and the RNAiRDR2 mutant. As expected, no differences between the two lines are apparent, and the correct display of the methylation state proves the bisulfite treatment was successful.

4. Discussion

4.1. Successful bisulfite treatment and amplification of a *Win1* promoter region

A 456bp long fragment (position 508-964) of the promoter region of the *S.tuberosum wound-induced1* gene was successfully amplified with PCR in both a wild type line and a mutant (RNAiRDR2) with reduced expression of the RDR2 protein. The DNA had previously been treated with bisulfite, and sequencing of a control region in a conserved exon of the tubulin gene proved that this procedure worked satisfactorily. These findings thus allowed for determination of the methylation state of cytosine residues.

4.2. WT and RNAiRDR2 show no considerable differences in CHH methylation

Sequencing of the bisulfite treated DNA in both lines resulted in precise determination of the methylation status of cytosine residues in different sequence contexts. While some differences could be observed between the two lines, these were not consistent when looked at over the whole sequence (WT with 4% lower CHH methylation than the mutant line) or when limited to the sequence corresponding to the *MiS23* insert (WT with 2% higher CHH methylation).

4.3. Conclusion

These findings do not provide data sound enough to confirm the hypothesis that the reduced siRNA levels in RNAiRDR2 would lead to lower CHH methylation in the *MiS23* insertion. While a trend toward lower methylation seems to be distinguishable at first sight when interpreting the graphical representation, one has to keep in mind that clones having the same methylation pattern are pooled. The calculated percentages are thus a better indicator of the actual methylation.

These unexpected results prompt questions on their origins. Unsuccessful bisulfite treatment is not a likely cause, since the controls described in chapter 2.6 showed that this step was successful. Also, the different cells within the organism do not necessarily show the same methylation state, as many environmental factors can influence the inducing mechanisms. It could thus be that the sequenced clones randomly showed similar methylation. This is however not very probable, as 16 (WT) and 15 (RNAiRDR2) clones were analyzed.

Assuming the present data if correct, another cause could be that the fragment that was analyzed within the scope of this project is not controlled exclusively by RdDM,

and that other mechanisms are responsible for its methylation, or that this fragment is simply not subject to RNA guided methylation.

Also, since the genomic DNA that was used in the present experiment was not identical to the one that had previously been sequenced and linked low CHH methylation to lower RNA levels, it could also be that the plant from which it was extracted was in another stage of growth, which could lead to other methylation behavior.

A number of follow-up experiments could help find a potential mistake that was made during this project, or determine whether the results presented here-in are factual. In order to do so, both bisulfite treated and untreated DNA should be used, and the current experiment be repeated, together with the previous experiments by the Baker lab that showed an implication of GATEs in RNA-mediated DNA methylation. This would not only prove whether the method is working, but also confirm the previous findings, and show that they were not due to special circumstances at the moment of DNA extraction (stress level of plant, siRNA levels, impurities) but can be generalized to a global phenomenon.

4.4. References

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Appendix

Table 11: Sequences of the primers used in this project.

X13497	LF1191	508-537	AtTTAACGATTTATGGAATGATGtTTGTTG	456
	LF1192	932-964	CTATTACTAATTAaCTTCACCATTTTCTCGAAA	
	LF91N	477-501	GTTAATGGTAATATTATGTGTTTTGT	499
	LF92N	952-976	AACAAAAATAACACTATTACTAATTA	
EU935742				
	LF981	3-29	caccGAGAGAAATttTTtAtATTtAAGGAGG	301
	LF982	280-304	TCCCAaCACCAaACTaTCCAAACAC	

Appendix 2: GenBank sequence of X13497 (*Win1* and *Win2)*

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1 gaattctgac tcaatcgaat tgagcaaatg gttgaaataa atagtataga gtagttaaat
61 gttatgaata taattaagat gattgtacgc gtaatcccac attatccatt tcaatacaat
121 atatgcaaaa atgaatgaaa ttagtaaatt ttggccatag atttcccaaa taatatttgg
181 gaaaaaattt ggcaaatagt gtttgtccat acagtttgtc attatttggc aaatattttt
241 ggcaaatatc ccaaattccc aaatactagt tttttctagt atttgggcca aatctcatta
301 tttgggatct tttgaaaatt aaaattttac cccaaacttt tatcttttac aaaaacatcc
361 tctatagtag ttgtttgtgt cgtattacat aatttttcac gtgaacacca aagtagtgat
421 gaaatattca gtgaatatta aatgatatgg ttgttgatga aaatgatgaa caataggcta
481 atggtaacaa agtcatgtgc tttgtctact taacgattta tggaatgatg cttgttgcac
541 tcactccaaa ctaccacatt gctctagtgt catgagcact atttgttatt gttgcaacga
601 agattcaatt gacttgtaat acaaacttat ggttagtttt gatagttttt aaaacttatg
661 ggtataaatc acatttttct aaaaaagtga aatatatttc ccaaatacta tggccaaaca
721 catagtgaaa tttcacccaa attttcaccc aaataatatt tggcaagaat atttagaaat
781 ctatggccaa acgctagctt aatatcccaa atttccaact caagttgtca aactaattaa
841 ttcagtctaa gtagctatta gctatcactt ttgaaaaaag gttctataaa aacccgcaag
901 taataagatc aaattatact actaattaat ctttcgagaa aatggtgaag ctaattagta
961 atagtactat tttgttatct ttgttcctct tcagcatcgc cgcaattgcc aacgcacagc
1021 aatgcggtag acaaaagggc ggtgccttat gcagcggcaa cttgtgttgc agccaattcg
1081 ggtggtgtgg gtcaacaccc gaattttgtt cacctagcca aggttgccag agccgctgca
1141 ctggtactgg tggatcaacc ccaactccat ctggtagcgc ccaaaacgtt cgtgcaacgt
1201 atcatatata caacccgcag aatgttgggt gggacctgaa tgctgttagt gcttactgct
1261 caacttggga tgctaataag cctttatcct ggcggaagaa atatggttgg actgctttct
1321 gtggtccagt cggacctcgt ggtcgagact cctgtggcaa gtgcttaagg gtacgtacga
1381 tctatttata taacaatatt tcattataat aaccaagttt tcgttcaaac tgacttttat
1441 atatatgtgc aggtgacaaa tacacgtaca ggagctcaaa cgacagtaag aatcgtggat
1501 caatgtagta acggaggact agacttggac gttaacgttt ttcgacaaat cgacacagac
1561 ggaaatggaa atcatcaagg ccatcttatt gtgaactacc agtttgttga ttgtggtgat
1621 aattaatctt atagttgaca cagaattata agattcatgt ttgactaccc aaataaatgt
1681 taaaagtatg aaatgaaaat aataataata ataataaagt tatttctttc tgcattttag
1741 aaaagtgaat tgatacaagt cacgtccaac aaattaaagt ccctcgtttg ttttatttaa
1801 ttaatgcaat gatattaatc gaacacaaaa ctaagattta atatgccgta tatataataa
1861 ttaaagaata tatatataaa tgtaaaatag tttctaatat tttgaaaaat gtccagaatt
1921 agacattatg ctcatataaa atgaaaagga aacaataaat ttctgggaag aggagggaaa
1981 gcaagactta gatgattcgg ataggggcgg aggcaacata tacttagggg gttcgaaacc
2041 ctcggcggaa aatattacta ctatttatat atggttaaaa ttatttttta ggtatgtata
2101 atagatgtcg aatccccttc ccctaattcg tgtgtttact tctcaaattt tgaaccccct
2161 tattaaaaat tctggctccg cactggattt agatatgtaa agaggaggtg taaagatgcg
2221 ttagtgagag gttggttgta gacttatagt agaagttagg aaaagtaaag gtagaccgaa
2281 atagagtatg tgtgtgttgg ggttagatat gatattatta accaatgtta atgctccaaa
2341 tattttatgg tacacttaat taaattataa gttgtttatc taaatatata taaatttatt
2401 tgcattgaat actttcttct taattaatgt aacactcata tataaaaatt tcaaacttcg
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2461 tgcatcatag agacaaacaa ataggataag atgcaaattt atgagtagta atattaaatt
2521 tcataatttg tgtggaggta taagcataat tccaaaaact ggtctgctag cctcatttat
2581 tcagctatac ctctattatc aacgcatttg tgggtcagaa gtagaaataa acttaagaaa
2641 ggtactttgt ctcttaatta ttcctactgt attttaattt taattttagt ttttatttaa
2701 aatatttgcc ttttctcatt atatagatat ataattttga tttatttatt tgaaaaacta
2761 tatcataatc aaatataaaa taattattga catactatat tggcacttaa atgatttagt
2821 atgtaattat tcattaaatt tatacaaaaa tttcataagt agatggatca aacatacgca
2881 tatcaagtaa aaaatgaagg tttgatgtat ttttttaaaa tagagatcac aaaaaaataa
2941 aagaaaaaaa atatcacgtg accataggga caccgaagtg tatttaaagt ttggctttct
3001 taaaggtccc tttttttaat tcaacgagga ggacttttтc tagatactta tcagaaacga
3061 cgctgtccaa caatttttтt ttttttgtt aatagccgcc tcaagtcgtg cacttctaaa
3121 agccgccatt agtttttatg gttttttttt cgattcgtgt tcgacattca tattagaatt
3181 caattgtatt tgaatttcca ttgtataaag ctttatttag gaaaattgct ccttatcaaa
3241 tatttttga aattataaaa tttaaaaata ttttttattt atttaaatac cttatttatg
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3361 atcctacggc ataaagtttt gacattctaa aaaataacac ctactccctc tgtccatttt
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3481 tacattaatt tgatatttta aataaaaaat ttaaatattc aaaaatcata cgaaaaatat
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3781 acacttttgg ataaaaaaaa atggttaagc taagttgtgg tcctatttta ctagctctgg
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4021 gccaaggggg cagcgcgcaa aacgttcgtg caacatatca tatatataac ccacagaatg
4081 ttgggtggga tttgaatgct gttagcgctt actgttctac ttgggatgct aataagcctt
4141 acgcctggcg gagtaagtat ggttggactg ctttttgtgg tccagtcgga cctcgtggtc
4201 gtgactcgtg cggaaagtgc ttaagggtaa gatatactag ctacatcctt ccgtttttat
4261 ttttagttga gaaaagttaa aattaaagag ttatcagaaa caacgaataa agtatatatt
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4381 caagtaagat tgctttacaa ctatgagttt gaatgagtac tggtcaacat tcactggttt
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