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HEAT STRESS TRIGGERS TRANSCRIPTIONAL ACTIVATION OF RNA-DIRECTED DNA METHYLATION PATHWAY GENES IN WHEAT AND BARLEY

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1 Abstract

Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are economically important crops that also have a crucial role in food security being among the most significant plants in humanity's calory intake.

As a consequence of climate change weather extremities are becoming the new norm and frequent heat waves jeopardize the yields of wheat and barley globally. Therefore, a better understanding of heat stress responses in wheat and barley is crucial to prepare for the decades of climate change to come.

RNA directed DNA methylation (RdDM) is a conserved pathway unique to plants that is able to regulate gene expression by methylating certain sequences in the genome. Key components of the RdDM pathway include Argonaute proteins (AGOs), Dicer-like proteins (DCLs), as well as nuclear RNA polymerase D (NRPD) proteins.

The literature on heat stress responses in wheat and barley is abundant, and functions of the mentioned proteins are also widely discussed, however no research on the expression levels of RdDM-related genes in heat stressed wheat and barley is available.

In this study, bioinformatic databases and software were used to identify or predict the sequences of these genes. To examine the phenotypic and gene activity changes in response to heat stress, multiple replicates of wheat and barley plants were subjected to heat stress. Gene expression levels were measured in a qPCR reaction and compared with control samples.

We hypothesized that four RdDM-related genes, namely *AGO6*, *DCL3*, *NRPD2A*, and *NRPD2B* would be upregulated under heat stress in wheat and barley. All four genes were upregulated in heat stressed plants when compared to control ones and most of these results were statistically significant. These results suggest that the RdDM-pathway has an active role in the heat stress response of these two important crops. Moreover, this response is conserved between the two plants.

This study provides new information on *NRPD2B* expression levels under heat stress conditions and is the first to experimentally compare wheat and barley in such circumstances, especially for the four genes of interest.

Our research could provide a basis for further experimentation to explain the genetic responses triggered by RdDM in more detail, as well as to provide data for the future breeding of heat tolerant wheat and barley cultivars.

2 Introduction

2.1 The significance of wheat and barley

Common wheat (*Triticum aestivum*) is a hexaploid plant species that people have been growing for millennia. Modern research shows that the plant's cultivation began in the Fertile Crescent, 8000-10000 years ago (Balfourier et al., 2019). This makes wheat one of the longest-cultivated crops in history along with barley (Ghahremaninejad et al., 2021).

Gruet et al. (2021) claim that, along with rice and maize, wheat is one of the most important crops globally, providing 20% of humanity's calory intake. In 2020, it was produced in 124 countries on 216 million hectares. Global wheat production is estimated to reach 780 million metric tonnes in the 2022/2023 season (World Agricultural Production). This is 55% more than the production of milled rice in the same period, and two-third of the production of maize (Statista, 2023), thus among crops mainly grown for human consumption, wheat is arguably the most significant.

Moreover, the overall dominance of wheat in humanity's calory intake is even more emphasised in more vulnerable social groups: Peña (2007) argues that wheat-based calory intake is especially high in rural and urban-poor parts of the population. This means that a bad yield and a following rise in the prices of wheat-based foods affects poor people more dramatically than less vulnerable parts of society.

Thus, achieving a certain level of stability in the yields of wheat production is of crucial importance not only for economic, but for social and food security reasons as well. However, the yields of wheat, as well as many other crops are threatened in their original cultivation areas by multiple challenges, one of them being unprecedented levels of heat stress.

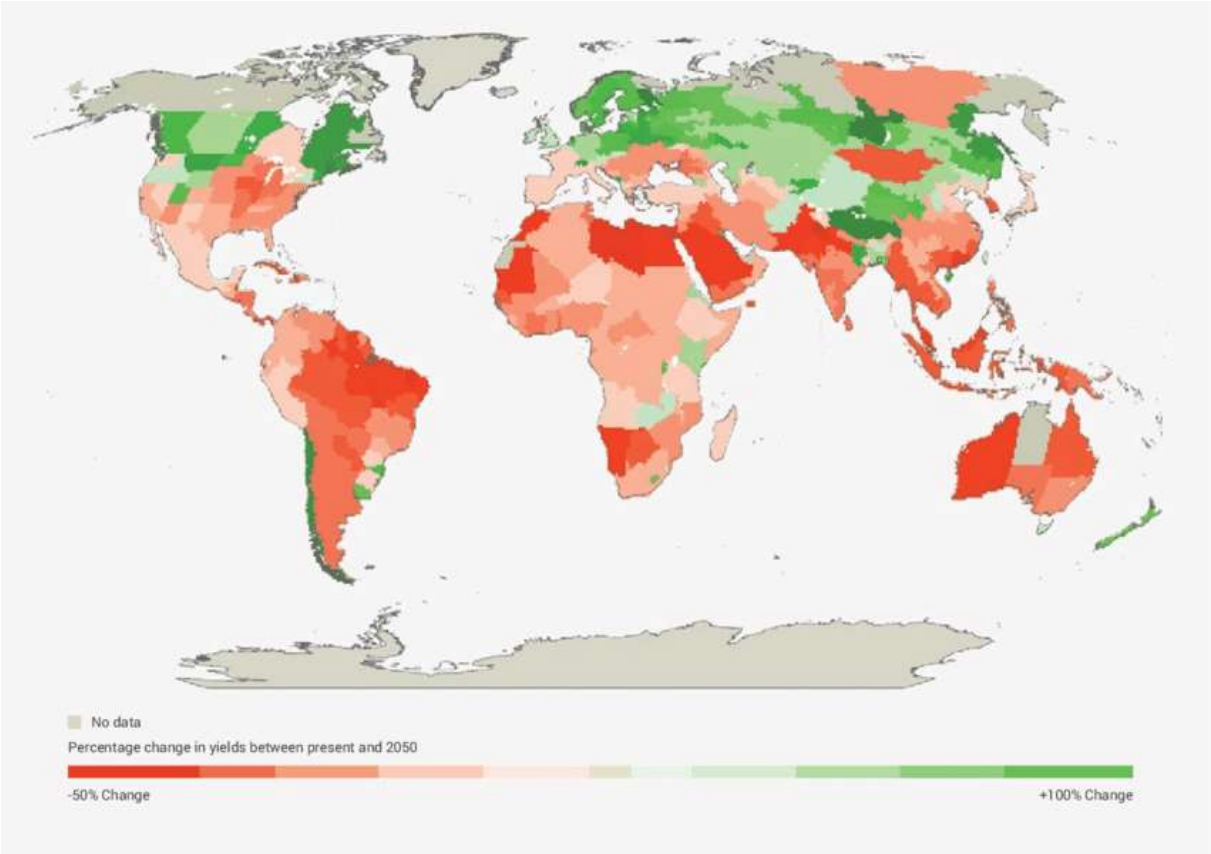
Barley (*Hordeum vulgare*) is also a crop that humanity has been growing for millennia. Today, its main use is for forage and malting, however, Baik and Ullrich (2008) argue that it used to be a predominantly food grain, and its main role changed during the centuries. Even today, barley is an important food grain in many regions, especially Asia, North Africa, the Middle East and Eastern Europe. Moreover, there is a growing interest in barley as a healthy alternative to refined grains, thus it could regain some of its former importance (Aldughpassi et al., 2016).

Global annual barley production averaged at 150 million metric tonnes between 2015 and 2020 (Tridge, 2022). The plant is cultivated on 50 million hectares, making it the fourth most important cereal in the world both by production volume and land use (Tricase et al., 2018).

2.2 The significance of heat stress

Climate change is altering many aspects of our lives and food production is no exception. If current trends continue, and cultivated plants keep being subjected to warming temperatures, a growing number of weather extremities and higher heat stress, that could have dramatic consequences.

The map below clearly shows that agricultural yields are expected to decrease for much of the Southern Hemisphere. This projected decrease is significant and could reach 50% in certain areas. While massive areas of the northern part of the Northern Hemisphere could experience an increase in yields, agriculturally important regions could suffer a reduction, including much of the USA, Mexico, Western, Southern and Eastern Europe, North Africa, and the Middle East.



1. *Picture: Percentage change in crop yields between 2013 and 2050 (Source: WRI, 2013)*

The fact that yields are expected to fall for most of South America, Africa, and Southeast Asia, the regions with the most challenging food security situations already further complicates the issue.

It is important to study the literature on the effects of heat stress on wheat and barley production thoroughly to assess the credibility of such predictions, as well as to prove the importance of breeding new varieties that are more tolerant to changing weather conditions and elevated heat

stress. These breeding processes can apply traditional practices; however, a better understanding of genetic pathways can also be beneficial.

Studying RNA interference and RNA directed DNA methylation pathways, the proteins participating in them, and how this process is altered by heat stress in wheat and barley could further our understanding of genetic responses to stress factors.

2.3 The significance of RNA interference and RNA-directed DNA Methylation

RNA interference is a cellular process through which small RNAs target and regulate complementary RNA transcripts, a mechanism that has well-characterised roles in post-transcriptional gene regulation and transposon repression (Gutbrod and Martienssen, 2020).

Sen and Blau (2006) assessed the most significant milestones of RNAi discovery and provided a brief history of this research field. They claim that an intricate network of proteins is the basis for this pathway, that ensures the degradation of target mRNAs.

They name the discovery of Napoli and Jorgensen as the first defining research in this field, where they experimented with petunias. The overexpression of the chalcone synthase gene in these plants resulted in white flowers, contrarily to violet ones that the researchers had expected. Romano and Macino (1992) achieved a similar result in their research *Neurospora crassa* where the introduction of a homologous RNA sequence silenced the endogenous gene.

Sen and Blau (2006) also mention the finding of Fire and Mello (1998) as a key discovery in RNAi research. Their hypothesis was that gene silencing was triggered by double-stranded RNA (dsRNA) rather than single-stranded RNA (ssRNA). Their assumption was that former successes with ssRNA-based gene silencing had been the result of dsRNA contamination. To test this theory, they used extensively purified sense and antisense ssRNA as well as dsRNA in another experiment. They found ssRNA to be 10-100 times less effective in silencing the same mRNA. The former was found to be effective only if both sense and antisense ssRNA was injected, suggesting an in vivo hybridization and the formation of dsRNA.

The discovery of RNA-directed DNA methylation (RdDM) followed a similar path: in the 1990s, many experiments were using transgenes to study their effects on host plants. In some cases, the transgene did not have an effect as significant as expected or had no phenotypical effect at all. Meyer and Heidmann (1994) introduced a transgene into petunias that was meant to increase the brick-red pigmentation of flowers. The transgene was integrated into an unmethylated genomic region, therefore should have been transcriptionally active. Instead, they found a decrease in pigmentation as a result of the methylation of the transgene DNA.

Early results like this suggest that RNAi and RdDM have an important role in post-transcriptional gene functions and gene silencing. Once aware of this fact, it is worth examining the role of gene silencing in heat-stressed plants, thus, in heats-stressed wheat and barley. Studying the literature on RNAi and RdDM in heat stressed plants might prove helpful in better understanding the genetics and stress responses of these socially and economically important crops.

2.4 Hypothesis

The goal of this study is to determine the activity of genes that take part in RNA-directed DNA methylation in heat stressed wheat and barley. The hypothesis is that *AGO6*, *DCL3*, *NRPD2A* and *NRPD2B* genes are upregulated and overexpressed in heat stressed wheat and barley, when compared to control plants.

3 Literature Review

The main focus of this study, RNA-directed DNA methylation is a process with similarities to RNAi, however the two are not identical. Erdmann and Picard (2020) define the notion as "a biological process in which non-coding RNA molecules direct the addition of DNA methylation to specific DNA sequences".

The literature on the role of RdDM in heat stressed organisms mainly includes *Arabidopsis thaliana* and *Caenorhabditis elegans*. The literature on plants and wheat or barley in particular is not extensive in this field. It is worth studying the literature of *Arabidopsis*, which, even though not closely related to wheat or barley, is a model plant of genetics and genomics and has a certain level of abundance in former research on heat stress and RdDM.

Popova et al. (2013) found heat stress to affect epigenetic gene silencing in *Arabidopsis*. They discovered that plants deficient in NRPD2, the common second-largest subunit of RNA polymerases IV and V were more sensitive to heat than plants in which this protein was functional.

Hamar et al. (2020) studied the activity of RdDM-related genes in barley in response to heat stress. Their results have a substantial relevance to this work, barley being within its focus and also being a monocot, thus more closely related to wheat then *Arabidopsis*. They call Dicer-like proteins (DCLs), Argonautes (AGOs) and RNA-dependent RNA polymerases (RDRs) the key components of RNA silencing. The former two are studied in this assessment as well. They identified five *DCL* and eleven *AGO* genes in the barley genome. They detected a transcriptional accumulation of small interfering RNA (siRNA) under heat stress, suggesting that RNA silencing is dynamically regulated and could be involved in the environmental adaptation of barley.

Zhao et al. (2020) claim DNA methylation happens through the transfer of a methyl group (CH_3) to cytosines to form 5-methylcytosine that is incorporated in a CG, CHG, or CHH complex (H representing A, T, or C).

Gallego-Bartolomé (2020) describes the role of AGO6 and DCL3 proteins in RdDM. After RNA polymerase IV synthesises single-stranded RNAs, and these RNAs are converted into double-stranded RNAs, DCL3 cuts these dsRNAs into 24-nucleotide small interfering RNAs (siRNAs). These siRNAs are then incorporated into AGO4 (as well as AGO6) proteins.

To induce the second part of this pathway, RNA polymerase V synthetises non-coding RNAs (ncRNAs) which are bound by siRNA-loaded AGO4/AGO6 proteins through sequence complementarity. As a result, an AGO-siRNA-ncRNA-Pol V ribonucleoprotein complex is

formed, followed by recruiting a domains rearranged methyltransferase 2 (DRM2) protein which targets DNA methylation. This pathway is unique to plants and has a crucial role in controlling gene functions (Erdmann and Picard, 2020).

Erdman and Picard (2020) describe the role of RdDM in stress responses in plants and specify that abiotic stress and heat stress in particular is also capable of triggering it. Under heat stress conditions, transposable elements (TEs) that are otherwise active can transpose into genes or promoters, which can have negative effects on the host genome. However, RNA-directed DNA methylation (RdDM) can silence these TEs and prevent them from transposing. They specifically claim that TE silencing is important in wheat, as it is rich in such regions and their silencing is crucial to genome stability.

The results of Korotko et al. (2021) might suggest the opposite of our hypothesis, as they found that the level of DNA methylation is decreased under the influence of heat stress in *Arabidopsis thaliana*. However, they found that the downregulation of methylation mainly appears in genes involved in heat stress response, thus allowing them to be active. Moreover, their stressing and sample-taking methods differ from those of this study. They heat stressed the plants at 42 ºC with no watering and they took samples 6 hours, 12 hours, and 24 hours after the end of heat stress, which is different from the experiment of this study (see Methodology).

Moreover, Boyko et al. (2010) claim that heat stress significantly increased DNA methylation in *Arabidopsis*, while Correia at al. (2013) found the same for cork oak (*Quercus suber*). In the former case, the plants were subjected to heat stress over a longer period of time, in the latter case, samples were taken during peak heat hours and immediately frozen until further use. This suggests that, if not given enough time to regenerate, heat stressed plants show an elevated level of methylation.

In their thorough literature review Zhao et al. (2020) also concluded that DNA methylation is involved in the epigenetic regulatory system in response to heat.

Popova et al. (2013) found through the analysis of DNA methylation-deficient mutants of *Arabidopsis* that the RdDM pathway is required for basal thermotolerance. They conclude that mutations in genes *NRPD2* (nuclear RNA polymerase D 2), *RDR2*, and *AGO4*, genes that are involved in the RdDM pathway, are hypersensitive to heat.

Akhter et al. (2021) assess that many gene sets are affected by heat exposure through changes in cytosine methylation, suggesting that genes related to methylation participate in responding to heat stress and developing tolerance.

Malabarba et al. (2021) identified a connection between DNA methylation and seed germination during seed development under heat stress. They analysed the dynamics of

transcript abundance during seed development under control conditions and heat stress conditions by whole-transcriptome RNA-Seq analysis in *Arabidopsis*. Their transcriptomic results showed differences in the expression of genes related to chromatin organization and DNA methylation on seeds upon heat stress. Their experiment considered different genes from those of this study, however, their results are relevant for this context. They found demethylase gene ROS1 to be downregulated as a result of severe heat stress. This suggests that *methylation* is a defence mechanism used by plants during heat stress, as they tend to avoid *demethylation*. The literature on RdDM and heat stress suggests that DNA methylation plays a crucial role in plants' response to abiotic stress including heats stress. Consequently, conducting experiments on further genes participating in RdDM and further plant species is relevant. The literature of this field justifies the analysis of *AGO6*, *DCL3*, *NRPD2A*, and *NRPD2B* expression levels in wheat and barley under heat stress.

4 Methodology

4.1 Identifying target proteins and their genes

Many proteins and many genes in wheat are not well annotated and therefore cannot be reliably labelled to be the protein or gene of interest just by knowing their sequences. This fact justifies the necessity of constructing phylogenetic trees that include sequences assumed to be proteins or genes of interest and similar sequences from better-annotated plant species.

Arabidopsis thaliana is a well-annotated plant species; therefore, its sequences can be effectively used to identify conservative sequences of interest in wheat. However, *Arabidopsis* is a dicot, thus, the also well annotated but monocot rice species *Oryza sativa* is more reliable when trying to identify less conservative sequences in wheat. These two species, along with barley (*Hordeum vulgare*) were used for the phylogenetic studies to identify AGO, DCL and NRPD proteins in wheat.

The two programmes used for this purpose were UniProt and Ensembl Plants. AGO4, AGO6, AGO15, DCL3, DCL5, NRPD2A, NRPD2B proteins were directly searched in UniProt, and the sequences found in *Arabidopsis* were than blasted in Ensembl Plants searching for rice, barley, and wheat. In cases where the protein's sequence was available and well-annotated in UniProt for rice as well, a blast was made for *Arabidopsis*, barley and wheat in Ensembl Plants using the sequence of rice.

The database of The James Hutton Institute was also used for identifying the expression levels of different genes in different development stages in barley. As these genes were not well annotated, not the name of the gene, but its sequence was used, and assumptions were made based on expression levels and well annotated genes in *Arabidopsis* and rice.

FGENESH+, an online gene finding tool was applied to improve the protein sequences of some of the wrongly annotated genes.

After clarifying the sequences and identifying genes of interest, the phylogenetic trees were constructed in MEGA11 software. The evolutionary history of genes of interest was inferred using the Neighbour-Joining method, showing the optimal tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. AGO proteins were analysed using 28 amino acid sequences. 14 sequences were used to analyse DCL proteins, and 18 sequences to analyse NRPD2 proteins. All ambiguous positions were removed

for each sequence pair (pairwise deletion option). There were a total of 1204 positions in the final dataset of AGO proteins, 2080 for DCLs, and 2206 for NRPD2s.

4.2 Plant growth and treatment

To test changes in gene functions and especially RdDM in heat stressed plants Golden Promise barley and Fielder wheat were sown on wet paper towels and kept in dark for three days. Next, the germinating plants were planted into Jiffy peat pellets. Prior to this, the pellets had been rehydrated using sterile water to avoid fungal infection. The young plants were then left to grow in these pellets to develop their roots. They were subsequently transferred into larger pots of soil to further develop their stems and leaves. Each plant was raised in a separate pot. They were kept well-watered for three weeks to gain enough strength to endure heat stress. During this period, a room-temperature environment was provided for them. More precisely, they were kept at 21 ^{o}C in a long-day conditioning process with 16 light hours and 8 dark hours.

Following this three-week period,12 wheat and 12 barley plants were divided into 4 replicates of 3 plants each and given a heat stress of 40 ^{o}C for 24 hours. The same amount of wheat and barley, i.e., 4 replicates of 3 plants each were kept at room temperature to provide a control. Their water supply was abundant for this day, so their reaction to heat stress could be examined without the additional effects of drought. The same number of plants was left at room temperature to serve as control for the experiment. After the 24 hours of heat stress, samples were taken from the leaves of both control and heat stressed plants and then stored at -80 ^{o}C until further use. Samples were taken from the apex of the youngest leaf on each plant.

4.3 RNA extraction

To observe gene activity in samples, their RNA had to be extracted. The leaves' cellular structure was broken by submerging them in liquid nitrogen in a mortar and smashing them with a pestle.

The extraction itself was the result of a phenol-chloroform method. The smashed leaves were first put in Eppendorf tubes of phenol. Heat stressed wheat and heat stressed barley, as well as control wheat and control barley samples were used, four each, which resulted sixteen samples in total. The samples were vortexed thoroughly, then put in a room temperature centrifuge at 14300 x g for five minutes. Following this centrifugation, the supernatant layer was put in new Eppendorf tubes containing phenol-chloroform. This mixture was also vortexed and centrifuged in the same manner. The resulting supernatant was put in Eppendorf tubes of chloroform this time and handled identically as the previous two.

After this, the supernatant was put in Eppendorf tubes with 99.8% ethanol in them, not vortexed but gently mixed by hand and put in -80 ºC freezer for 1 hour, then centrifuged at 4 ºC for 30 minutes at max speed. The ethanol was then poured off to keep the pellet of the nucleic acids and the tubes were washed with 70% ethanol. This was once again centrifuged at room temperature for 5 minutes. The 70% ethanol was also poured off and the open tubes were put in a SpeedVac (vacuum concentrator) for 10 minutes to completely dry out so that only the nucleic acid pellets remain in them. The nucleic acid was then dissolved by putting water in the Eppendorf tubes and vortexing them.

The Eppendorf tubes containing the samples were kept on ice during the whole process when they were not being processed in some way. This was necessary to avoid RNA degradation.

4.4 Designing primers

Primers were designed in Primer3 software to be common between wheat and barley when possible. Another important goal was for the primers to be located on the exon-exon junction to avoid genomic DNA contamination in PCR reactions. Avoiding secondary structures, such as complementarity and hairpin was also among the designing aspects.

Melting temperatures were also set to remain within a certain range to give clearer results in PCR. The same *AGO6* primers were designed for wheat and barley with an amplification length of 109 base pairs and an annealing temperature of 65.1 ºC. For *DCL3*, different primers were designed for the two plants. For wheat, it was a primer with an amplification length of 160 base pairs and an annelaing temperature of 64.7 ºC. The *DCL3* primer of barley had a 168 base pair amplification length with an annealing temperature of 64.3 ºC. *NRPD2A* and *NRPD2B* also had common primers for wheat and barley, the former amplifying a sequence of 116 base pairs, the latter having an amplicon length of 102 base pairs. Their annealing temperatures were 64.8 °C and 64.4 °C respectively. The primers designed for the reference gene, actin amplified a region of 161 base pairs and had an annealing temperature of 62.8 ºC in wheat, while they amplified a region of 181 base pairs and had an annealing temperature of 64.2 ºC in barley.

The table below shows the exact sequences of the primers used in the qPCR reaction. It also contains the names and amplicon lengths of primers.

Gene Name	Primer Name	Sequence (5'-3')	Amplicon Length
HvActin	HvActin_qPCR_F	ATGTTCCCAGGTATCGCTGAC	181 bp
	HvActin_qPCR_R	ACTCGTCGTACTCATCCTTGG	
TaActin	TaActin_qPCR_F	AGACTTTCAATGTTCCTGCCATG	161 bp
	TaActin_qPCR_R	CAAACGAAGAATGGCATGAGG	
HvAGO6/ TaAGO6	Hv-TaAGO6_qPCR_F	CAGGGATGGTGTGAGTGAGTC	109 bp
	Hv-TaAGO6_qPCR_R	TGTAATCTTTGGAGGTGGCCC	
HvDCL3	HvDCL3_qPCR_F	CTTCCAGGGGACAAGTACGAC	168 bp
	HvDCL3_qPCR_R	TGCATCAAGACAAACGAGCTG	
TaDCL3	TaDCL3_qPCR_F	TGAGAGTGGATGTGTGGAAGC	160 bp
	TaDCL3_qPCR_R	CTTCATTGTCCGTTCCATGGC	
HvNRPD2A/ TaNRPD2A	Hv-TaNRPD2A_qPCR_F	GCGTTTAAGGTGCATGAGACG	116 bp
	Hv-TaNRPD2A_qPCR_R	TGAGTAATCGCCCTGCATCAG	
HvNRPD2B/ TaNRPD2B	Hv-TaNRPD2B_qPCR_F	GATTTGGTTGGTGTGAGTGCA	102 bp
	Hv-TaNRPD2B_qPCR_R	GTCCCTTTTGATTTCCACCTGTG	

1. Table: Primer sequences and amplicon lengths

4.5 DNase treatment and cDNA synthesis

As the goal was to observe gene activity, DNA had to be removed from these samples, containing all nucleic acids, leaving only RNA behind, from which, cDNA could be synthesized.

For this re-isolation a method, similar with the one described in Extraction was used, however, in this phenol-chloroform protocol, pure phenol was omitted, and only phenol-chloroform and chloroform were used. The remaining DNA was digested using 1 µL of DNase I NEB by treating it at 37 ºC for 60 minutes. After this, 2 µL of RNA solution containing 500 ng of RNA was run on agarose gel to check RNA quantity and remaining DNA contamination.

Following this isolation step, 4000 ng of RNA was dissolved in 22 µL of water. 11 µL of this containing 2000 ng of RNA was used for cDNA synthesis. For the synthesis of cDNA, a RevertAid RT Reverse Transcription Kit was used. This contained oligo dT primers to amplify mRNAs, as the goal was to observe gene activity.

4.6 RT-qPCR

Quantitative reverse transcription polymerase chain reactions, (RT-qPCRs) were used to determine the expression level of *AGO6*, *DCL3*, *NRPD2A* and *NRPD2B* genes in heat stressed and control plants.

The master mix used for these reactions was Luminaris Color HiGreen qPCR Master Mix. The samples were loaded in a Roche LightCycler® 96 System.

Actin was used as a reference gene, as its expression shows a high level of stability in heat stressed plants compared to control ones (QianYing et al., 2019). This was also tested in a gel electrophoresis where Actin PCR-amplified Actin genes and PCR-amplified *NRPD2B* genes were run on a gel. The Delta-Delta CT-method was used to define the expression level of target genes.

The software used to depict the results of the qPCR and the expression levels was GraphPad Prism 8. An independent T-Test was used to determine the significance of the results.

5 Results

The results are divided into phylogeny, heat stress response and gene activity chapters, as these were the fields of research that provided the highest level of novelty and best answered our hypothesis.

5.1 Phylogeny

After completing the phylogenetic research described in 4.1, the resulting phylogenetic trees were of great help to better understand the evolutionary relations between genes of interest and closely related genes, as well as to make annotations more reliable and identify or at least assume the sequences to be AGO6, DCL3, NRPD2A, and NRPD2B.

5.1.1 AGO6

The result of the phylogenetic research on AGO proteins described in 4.1 is the phylogenetic tree below (Figure 1). The tree is showcasing certain proteins of the AGO4-6 clade and their evolutionary relationships to each other.

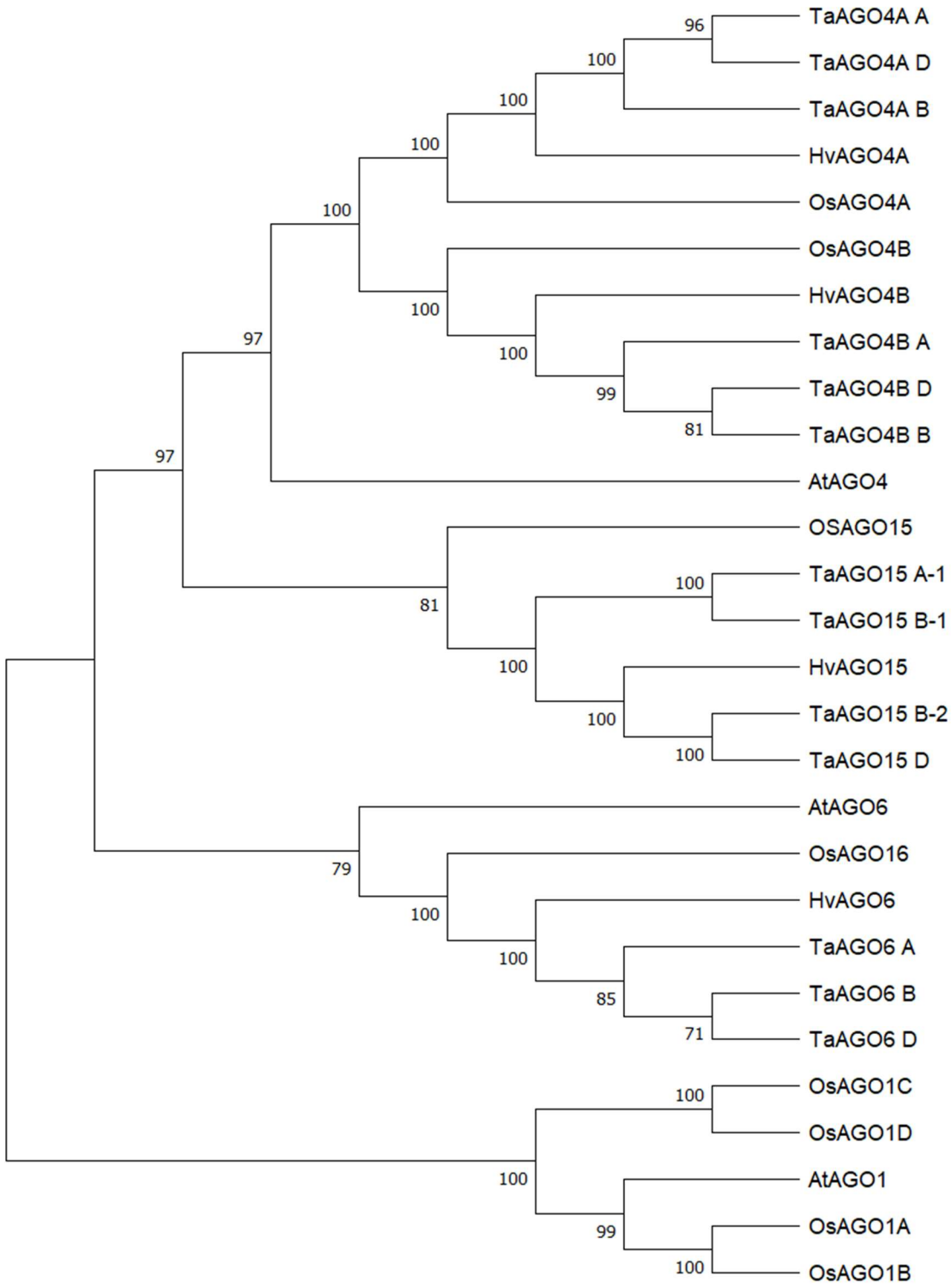
Only the abbreviations of the species' Latin names and the names of the proteins are shown on the branches, however, if the full annotation of the proteins is considered along with our results as to which protein is found on which branch, the evolutionary relations between these plants and their proteins can be better understood. The full annotation shows that genes for the protein *AGO4A* are located on the 3rd chromosome of all three wheat genomes as well as on the 3rd one of the barley genome. Similarly, all the genes of the protein *AGO4B* are located on the 1st chromosome of all three wheat genomes and the first one of barley. Synteny provides an explanation for this. Britannica describes synteny as a situation where organisms of relatively recent divergence show similar blocks of genes in the same relative positions in the genome and possess common chromosome sequences. Bennetzen and Freeling (1997) suggest that, even though the monocot/dicot divergence is around three times more ancient than the last common ancestor of grasses is, regions of synteny can be observed even between monocots and dicots. This suggests a much higher level of synteny within the family of grasses. Moreover, Swarts et al. (2014) claim Argonaute proteins to be conserved across all domains of life, meaning they are highly conservative. These two facts explain the similar position of *AGO4* genes on barley and the three wheat genomes. Considering all three monocots in the tree (rice, wheat, and barley), a closer evolutional relationship is visible between *AGO4A* genes of different species, than between *AGO4A* and *AGO4B* genes of the same species. This is also the result of synteny and the highly conserved nature of the proteins and their genes.

As for the only dicot in this phylogenetic tree, the *AGO4* gene of Arabidopsis was further off the same branch of the tree, indicating a more distant evolutional connection.

AGO15 was also put in the tree to achieve a more complete picture. Trujillo et al. (2018) call *AGO4A* and *AGO15* sister groups that share high sequence similarity and are located within a few kilobases from each other. On the other hand, Liao et al. (2020) found that AGO15 differs from the other paralogs, as it has residues of different physico-chemical properties at functionally important amino acid positions. AGO15 also showed close relations between the monocot plants of the tree.

Results were similar for the gene of *AGO6*. Genes in the barley genome and the three wheat genomes were closely related, with the third monocot also not too far-off (the gene *AGO16* in rice can be considered the closest relative of *AGO6* genes in wheat and barley, *AGO16* being only the name of the gene, while it is considered an AGO6 clade protein). Arabidopsis was once again further away, being a dicot.

The visualization of Shevala (2023) on Argonautes explains why AGO6 only has one sequence in analysed plants, and why the protein from the same clade, AGO4 has two sequences in monocots (AGO4A, AGO4B). While AGO6 is highly conserved through angiosperms, the AGO4 clade expanded in grasses. AGO4 only has one gene in *Arabidopsis* because of this late expansion.



1. Figure: Neighbour-Joining Phylogenetic tree of selected AGO4-6 clade proteins in wheat, barley, rice, and Arabidopsis

The activity of *AGO1* genes was not a focus of this research. Sequences of AGO1 were added to the tree as an outgroup, meaning a taxon outside the group of interest in order to create a separate branch system that contains all the AGO4 and AGO6 sequences in the examined plants.

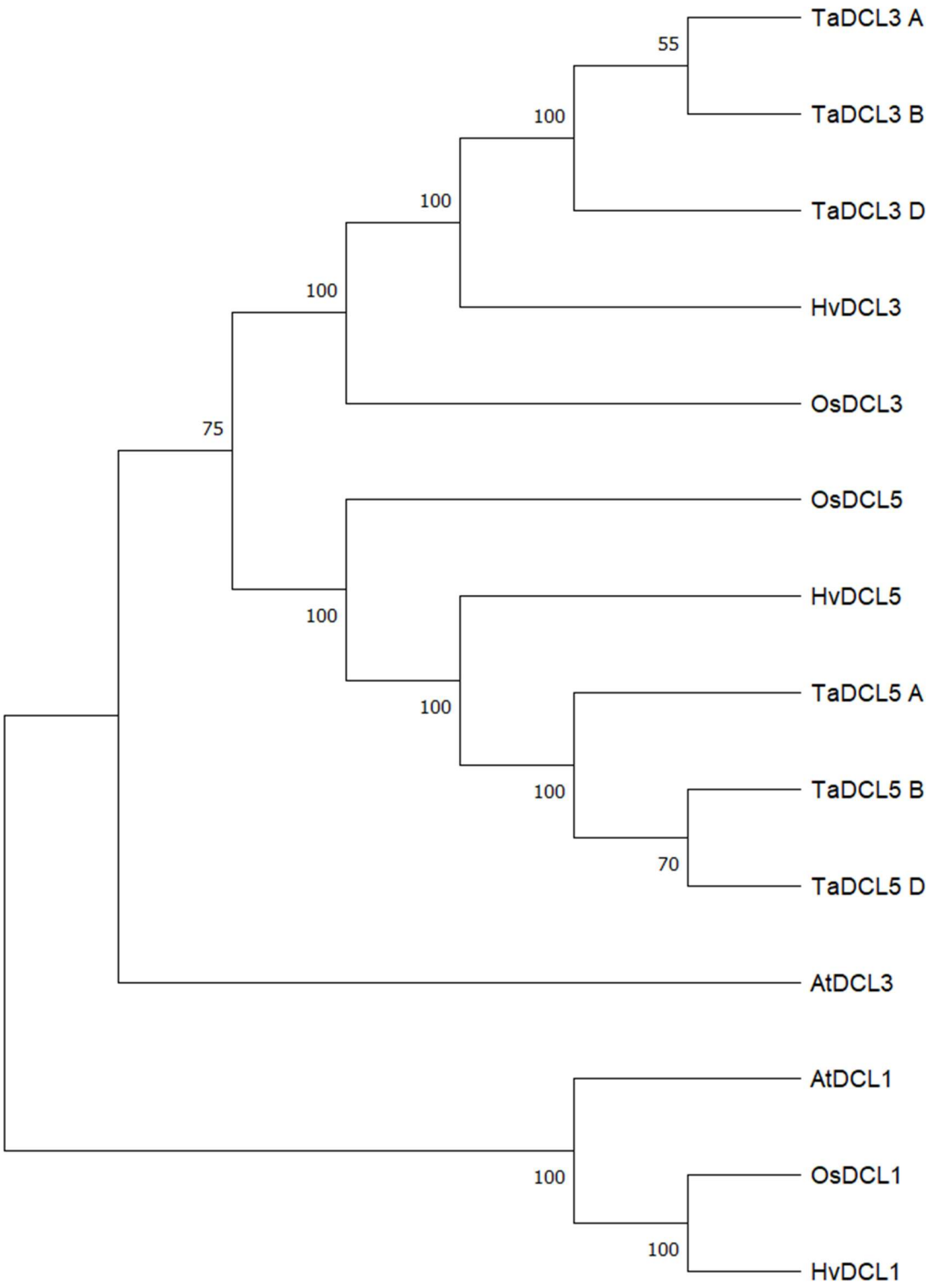
Since the members of the group of interest are more closely related to each other than they are to the outgroup, the outgroup stems from the base of the tree.

5.1.2 DCL3

The result of the phylogenetic research on DCL proteins described in 4.1 is the phylogenetic tree below.

A tendency similar to the one of *AGO* genes is observed on this tree. *DCL3* genes of wheat genomes and other monocots were closely related, more so than *DCL3* and *DCL5* genes in the same species. DCL1 sequences were added, similarly to AGO1 sequences in the AGO-tree to create a separate branch system that contains all DCL3 proteins along with DCL5 sequences.

The tree contains both DCL3 and DCL5 proteins for all monocots, but only DCL3 and DCL1 for *Arabidopsis*. Chen et al. (2022) analyse how *DCL3* and *DCL5* have become functionally specialized after gene duplication. This means that *DCL5* is a result of the duplication and mutation of the *DCL3* gene that have likely appeared in monocots.

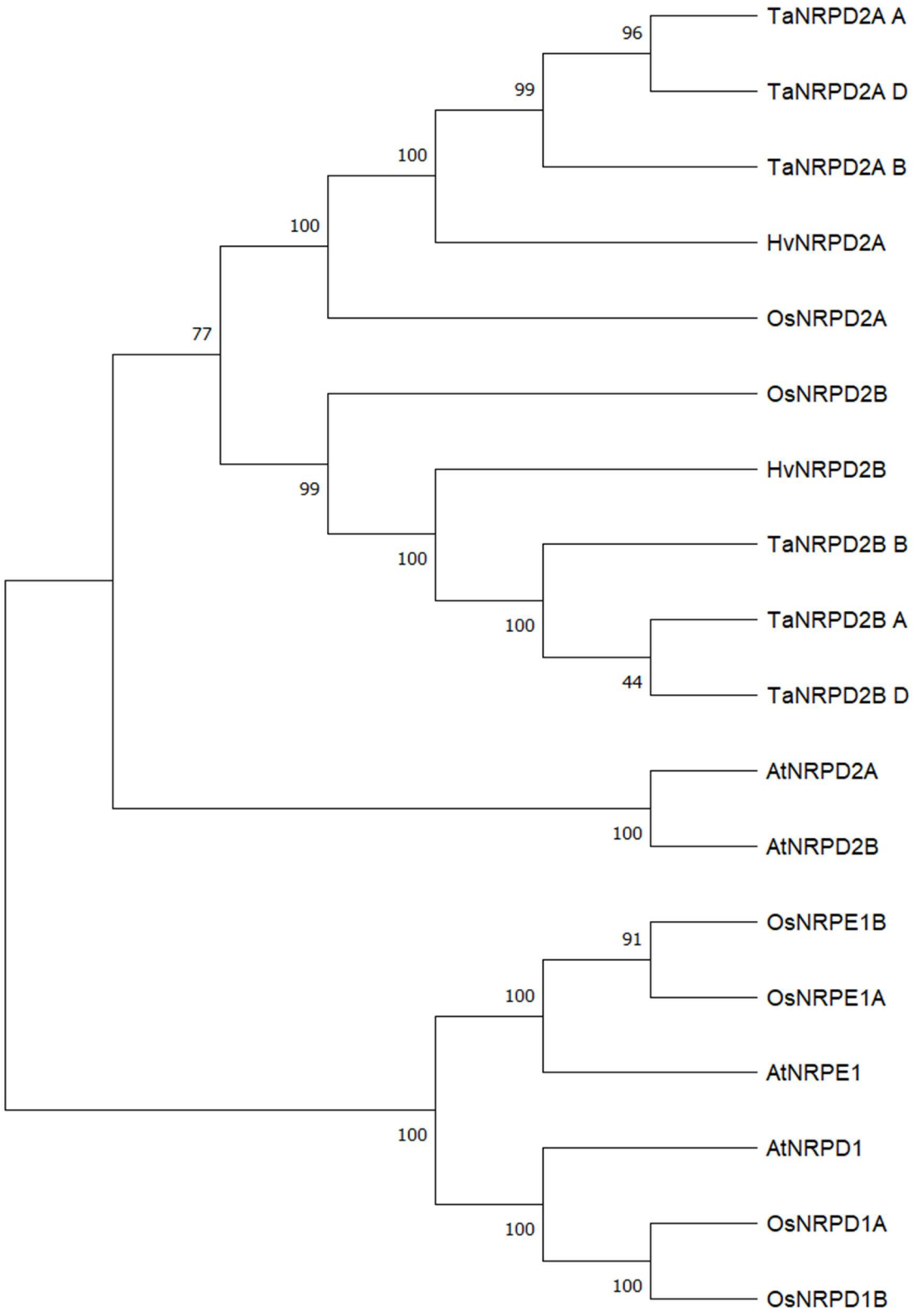


2. Figure: Phylogenetic tree of selected DCL proteins

5.1.3 NRPD2

The result of the phylogenetic research on NRPD2 proteins described in 4.1 is the following phylogenetic tree. The database of The James Hutton Institute was especially helpful in identifying *NRPD2* genes. As the second largest common subunit of RNA polymerase IV and

V, *NRPD2A* had higher expression levels in *Arabidopsis* than *NRPD2B* This information led to the assumption that the *NRPD2* sequence with higher expression levels is *NRPD2A* and the one with lower expression levels is *NRPD2B* in barley.



3. Figure: Phylogenetic tree of selected NRPD proteins

The phylogenetic trees of AGO and DCL sequences contained only one gene of interest each, i.e. *AGO6* and *DCL3*. The remaining sequences were added only to increase the reliability of the analysis and to better visualize the content. Only *AGO6* and *DCL3* were the ones in the focus of this study, the expression levels of which were investigated during the research.

For *NRPD2*, two sequences, however closely related were analysed for their expression level, both of them also present in the phylogenetic tree above. The reason for this is the shared function of NRPD2A and NRPD2B as the second largest common subunit of RNA polymerase IV and V. This function puts them both in the focus of this study. AGO and DCL proteins other than AGO6 and DCL3, as closely related as they may be to these two, do not have the exact same role and were thus only added for the reasons described above. Therefore, in this tree, two sequences were in the focus, and two (NRPD1, NRPE1) were added for reliability and visualisation.

The tendency was once again similar to the ones previously observed. NRPD2A sequences of the three wheat genomes and barley were relatively close to each other and not much further from the analogous sequence of rice. NRPD2B was further away from NRPD2A, but monocots were once again close to each other. The dicot Arabidopsis was further for both NRPD2A and NRPD2B. NRPD1 and NRPE1 created a separate branch system, and these were only added for rice and Arabidopsis, as their expression levels in wheat and barley were not examined in this study, therefore, their purpose was to provide better visualisation.

5.2 Heat stress response

The heat stress process resulted in 12 heat stressed wheat and 12 heat stressed barley plants, while the same number of plants were left under control conditions. Following the heat stressing and sample taking processes, photos were taken of the heat stressed plants and the control ones next to each other. Heat stressed plants can be seen on the right, control plants are on the left (Fig. 4 and 5.). At this point, the expression levels of genes of interest (GOIs) were not know, however, visible results already showed significant changes as a result of heat stress.

As the pictures clearly show, heat stress had a significant effect on both wheat and barley. 24 hours at 40 ^{o}C visibly decreased the turgor of all heat stressed plants. Moreover, wheat plants seem more affected, as their turgor decreased more profoundly than that of barley plants; a result perhaps unexpected, given that barley is generally grown in milder, while wheat is grown in warmer climate. The clear phenotypical changes suggest genotypical changes as well, the observation of the latter being the focus of this study.

4. Figure: The effect of heat stress on wheat directly after stress period

5. Figure: The effect of heat stress on barley directly after stress period

The next two pictures show the plants in the same orientation after one week of recovery. The stronger turgor in wheat might be part of the reason why it was more visibly affected by heat stress directly after treatment.

6. Figure: Control and heat stressed wheat plants after one week of recovery

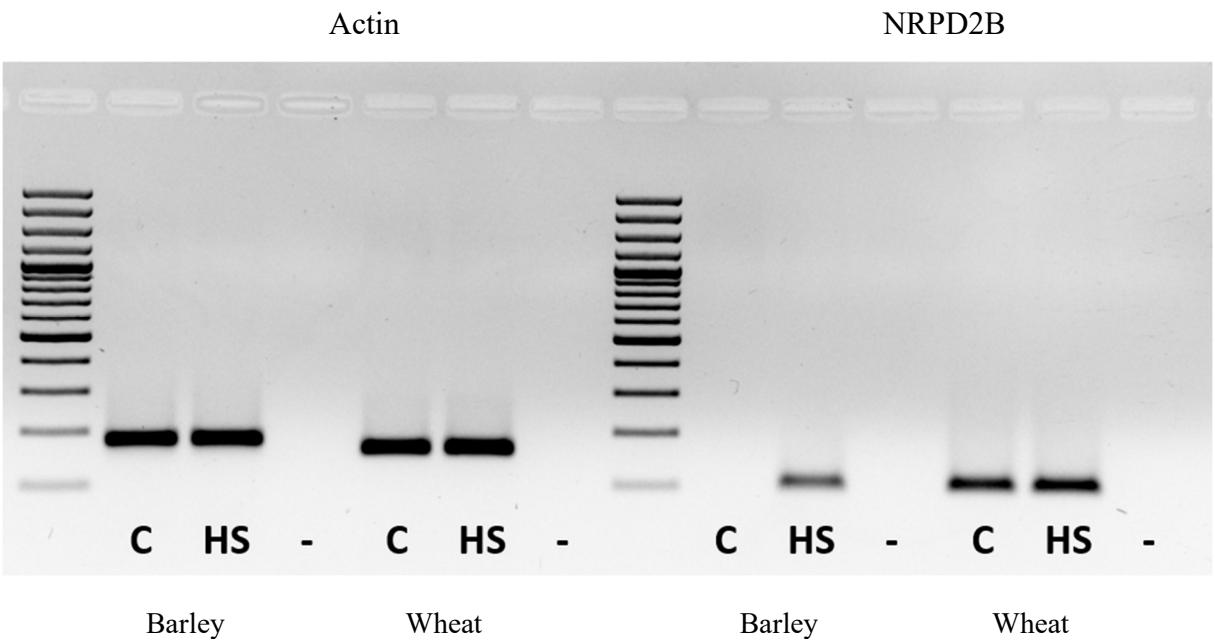
7. Figure: Control and heat stressed barley plants after one week of recovery

5.3 Gene activity

5.3.1 Actin as a reference gene

Our aim is to evaluate some of the genes that are involved in the RdDM during heat stress for understanding their responses.

As mentioned in 4.6, the ability of Actin to serve as a reference gene was not only based on relevant literature, but it was also tested in a gel electrophoresis using a semi-quantitative PCR.

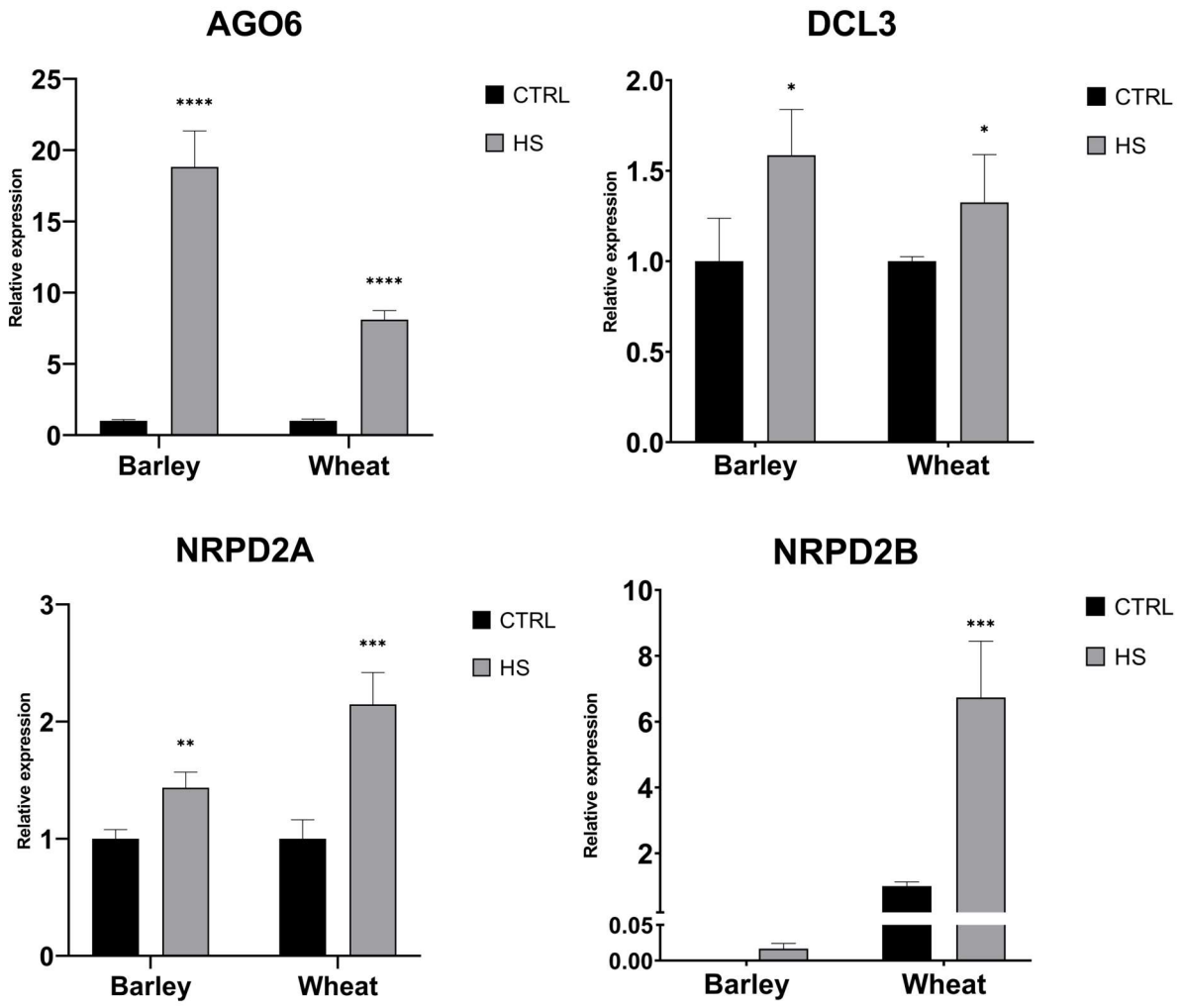


8. Figure: Gel electrophoresis of Actin and NRPD2B

As the picture shows, there was no visible difference in the expression level of actin genes in heat stressed wheat and barley when compared to control plants. The same cannot be said about *NRPD2B* (the lack of expression in control barley complicated qPCR for this gene, see in 5.3.2). This stability is an essential quality of a reference gene for a Delta-Delta CT-method qPCR.

5.3.2 qPCR

The Delta-Delta CT-method is able to show the expression level of genes compared to other genes in the same reaction. The number of cycles that enable the user to detect the Luminaris fluorescent signal in a sample is not very informative on its own, but highly informative when compared to other samples. As the number of cDNAs is doubled in each cycle, the fewer cycles it takes for the machine to detect the signal, the more cDNA the sample originally had. If a Sample 1 takes X cycles to be detected and Sample to takes X+3 cycles to be detected, than Sample 1 had 2^3=8 times more cDNA originally. Using this method, the following results were observed.



9. Figure: Expression levels of genes involved in RdDM in wheat and barley

**: P-value<0.05; **: P-value<0.01; ***: P-value<0.001; ****: P-value<0.0001*

As clearly visible from the figures, results are mostly significant for all four genes, however, to a different extent. Stars represent a the level of significance using the P-value (the chance of our null hypothesis to be true).

AGO6 expression levels were significantly upregulated for both wheat and barley. In heat stressed barley samples, the expression level of this gene was on average 18 times higher than in control ones. As for wheat, heat stressed samples' *AGO6* content was 9-fold higher than that of control samples, although with a lower level of standard deviation, resulting in a similar level of significance.

The lowest expressional difference was observed for *DCL3* in both wheat and barley, although results were still significant. For barley, expression levels were on average 1.5 times higher in heat stressed samples than in control ones, while heat stressed wheat samples displayed an expression level 1.3 times higher on average.

NRPD2A genes showcased a two-star level of significance for barley and a three-star level of significance for wheat. The gene on average 1.5 times more expressed in heat stressed barley and 2.1 times more expressed in heat stressed wheat than in control samples.

The qPCR of *NRPD2B* had a unique result, as no gene activity was detected in control barley, as visible in Figure 8 too. Therefore, the expression level of heat stressed barley was normalised on control wheat samples, as it could not be normalised on control barley with a value of 0. Nonetheless, *NRPD2B* was detectable in heat stressed barley, unlike in control samples, meaning that a gene activation is visible, even though with values much lower than in wheat, and wheat used as a control. In wheat, *NRPD2B* was almost 7 times more expressed under heat stress than under control circumstances, resulting in a three-star significance level, or 99.9% for the hypothesis to stand.

The chart below is an overview of heat stressed samples' average expression levels for genes of interest, relative to control samples (control sample=1). For *NRPD2B* in barley, the value of control wheat equals 1*. It also shows the probability that the hypothesis is true, i.e., genes are overexpressed in heat stressed plants when compared to control ones.

	AGO6 HS		DCL3 HS		NRPD2A HS		NRPD2B HS	
	Relative expression	P-value	Relative expression	P-value	Relative expression	P-value	Relative expression	P-value
Barley	18	0.0001	1.5	0.05	1.5	0.01	0.02*	n. a.
Wheat	9	0.0001	1.3	0.05	2.1	0.001	7	0.001

2. Table: Relative expression levels in heat stressed samples (C=1), and their significance level

Any level of expression after a heat stress treatment would be biologically significant, even if it cannot be proved to be connected to heat stress. However, these results are statistically significant for all genes in both plants, except for *NRPD2B* in barley, meaning that it is likely (likeliness is described by the P-value) that the upregulation is the result of heat stress.

6 Discussion

The results confirm the hypothesis of this study: *AGO6*, *DCL3*, *NRPD2A*, and *NRPD2B* genes are upregulated and/or activated in heat stressed wheat and barley, when compared to control plants.

The phylogenetic research provided some results worth discussing. In the case of the AGO4-6 clade, it is possible that the duplication of AGO4 appeared in a common progenitor, and the division between the two sub-clades in monocots can be observed for this reason. The phylogenetic study of NRPD2 also gave a result that requires explanation. Sequences of NRPD2A and NRPD2B in Arabidopsis are together, separately from orthologous sequences of the monocots. There are multiple possible explanations for this. One is that the duplication appeared independently in dicots and monocots. Another is that more and more differences accumulated during the evolution, resulting in separation.

As described in 5.2, wheat produced a more visible phenotypical reaction after 24 hours of heat stress, even though it is generally grown in warmer climate conditions. This might be explained by the fact that wheat adapts to heat faster and applies defence mechanisms, such as decreasing evaporation more effectively, while the same takes more time for barley, being less adopted to heat. This is what the pictures taken after recovery suggest as well (Figure 6 and 7).

As for qPCR, an important finding is that, apart from *NRPD2B*, the significance of upregulation was relatively similar in wheat and barley for the same gene. The largest difference was present in *NRPD2A*, and even there, the P-value for barley was <0.01, while the P-value for wheat was <0.001. For *AGO6* and *DCL3*, the significance of the results was almost identical in wheat and barley.

For *AGO6* and *DCL3*, a higher average upregulation and a higher standard deviation of the data was present in barley, while for *NRPD2A*, the contrary was true. For *NRPD2B*, the upregulation and significance were more reliably measured in wheat, however, the result of barley might be more interesting, as there was no detectable expression in control samples, while expression was measured in heat stress plants. This means that the gene was *activated* in barley under heat stress.

6.1 Explanation of results

Considering the literature review that clarifies the importance of these proteins in RNA regulated DNA methylation, and our results, it can be assessed that RdDM activity is increased as a result of heat stress in two economically important crops, wheat and barley.

As Erdmann and Picard (2020) claim, RdDM is a pathway unique to plants, and abiotic stress including heat stress might explain the evolutionary relevance of this process and its exclusiveness to plants. By methylating certain genes, RdDM changes physiological processes for a longer period of time than RNAi does. This is crucial for plants, being immobile and having to adopt to changes in their environment without relocating. While the short-term RNAi pathway might prove sufficient in animals, as, for them, gene silencing is only required until they move to an optimal environment, plants need a solution that lasts, but can still be reversed if their surroundings change.

6.2 The novelty of this study

As the literature review shows, multiple studies have studied RdDM pathways in several plants. In this study, the activity of genes of the RdDM pathway have been examined in wheat and barley together, an aspect was not previously present in the literature. This combined research enabled the authors to make comparisons between the two plants based on the activity of different genes.

Another novelty of this study is a deeper analysis and expression measurement of *NRPD2B* in heat stressed wheat and barley. The other three genes in the focus of this study have been more widely analysed under heat stress conditions and in both plants as well. As far as our thorough literature review is concerned, expression levels of *NRPD2B* have not been analysed in heat stressed wheat or barley before. Moreover, this is the gene that arguably provided the most interesting result: a high level of upregulation in heat stressed wheat; no expression in control barley plants and activation in heat stressed ones.

6.3 Suggestions for further research

The results of this study open the door for a wide range of new research in the field of RdDM, and heat stress in plants.

The results suggest that RdDM activity is increased in wheat and barley under heat stress, however, this study did not examine which part of the genome have been methylated by this response. This could be a new area of research, that would show which genes are silenced by methylation under heat stress.

Another area, that is derived from the first one is the one of phenotypical change. Learning which genes are methylated could provide a basis for the observation of changes in the phenotype of plants and what physiological processes are triggered by the silencing of certain genes.

The combined results of these two research areas could provide a better understanding of which genes are methylated under heat stress, what are the consequences of this silencing for the plants' physiology, and how do these changes manifest in phenotypes. A certain level of phenotypical response has been observed in this study (see 5.2), however, a more detailed examination of, and a deeper understanding of the genetics behind these changes could prove to be valuable knowledge.

Finally, a more complex picture of heat stress response, that includes the results of this study, as well as future results described above could enable researchers to interfere in these pathways and develop new breeds that are more resistant to heat stress. This interference could be based on biotechnological methods, especially genetic modification, and traditional breeding processes, or the combination of the two, such as MAS (marker assisted selection). The new breeds would have a high and increasing relevance in terms of food security and economic feasibility in the following decades of climate change.

7 Conclusion

Wheat and barley are globally important crops, both in terms of food security and economic value. Climate change threatens the yields of these crops, and, along with other weather extremities, heat stress is among the most important factors of this process.

This study aimed to achieve a better understanding of heat stress responses in wheat and barley on a molecular level with a special focus on RNA directed DNA methylation and the genes participating in it. To establish this research, the literature of heat stress responses in wheat and barley, as well as RdDM have been thoroughly studied.

To identify the genes of interest, i.e., *AGO6*, *DCL3*, *NRPD2A*, and *NRPD2B*, online databases and bioinformatical software were used, and phylogenetic trees were constructed. Apart from making previous assumptions on the nature of not well-annotated sequences more reliable, these trees also gave an insight into the evolutionary relations between the genes and plants included.

Heat stress had a more severe immediate effect on wheat, however, it also showed a fuller recovery after one week than barley, possibly because the former is generally grown in warmer climate conditions, therefore more adaptive to heat than the latter.

The results of the PCR show that all four of the GOIs were either overexpressed or activated under heat stress in both wheat and barley. These results were statistically significant, confirming our hypothesis: *AGO6*, *DCL3*, *NRPD2A* and *NRPD2B* genes are upregulated and overexpressed in heat stressed wheat and barley, when compared to control plants.

The research resulted in a certain level of novelty in the comparison of heat stress responses in wheat and barley, more precisely, the experimental comparison of the two species together under heat stress. Another novelty was the observation of *NRPD2B* expression levels in heat stressed plants.

Our results could encourage further research in the field of genetic and genomic changes, along with physiological and phenotypic modifications under heat stress. This could subsequently provide a scientific basis for biotechnological modifications of these plants that enable them to be more resistant to new challenges posed by climate change.

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9.2 Online Databases

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Downloaded: 09.10.2023

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Figures and Tables

NYILATKOZAT

a diplomadolgozat nyilvános hozzáféréséről és eredetiségéről

A hallgató neve:	Basa Márton
A Hallgató Neptun kódja:	CSIBR7
A dolgozat címe:	Heat Stress Triggers Transcriptional Activation of RNA-Directed DNA Methylation Pathway Genes in Wheat and Barley
A megjelenés éve:	2023
A konzulens intézetének neve:	Genetika és Biotechnológia Intézet
A konzulens tanszékének a neve:	MATE GBI Növénybiotechnológiai Tanszék

Kijelentem, hogy az általam benyújtott diplomadolgozat egyéni, eredeti jellegű, saját szellemi alkotásom. Azon részeket, melyeket más szerzők munkájából vettem át, egyértelműen megjelöltem, és az irodalomjegyzékben szerepeltettem.

Ha a fenti nyilatkozattal valótlant állítottam, tudomásul veszem, hogy a záróvizsga-bizottság a záróvizsgából kizár és záróvizsgát csak új dolgozat készítése után tehetek.

A leadott dolgozat, mely PDF dokumentum, szerkesztését nem, megtekintését és nyomtatását engedélyezem.

Tudomásul veszem, hogy az általam készített dolgozatra, mint szellemi alkotás felhasználására, hasznosítására a Magyar Agrár- és Élettudományi Egyetem mindenkori szellemitulajdon-kezelési szabályzatában megfogalmazottak érvényesek.

Tudomásul veszem, hogy dolgozatom elektronikus változata feltöltésre kerül a Magyar Agrár- és Élettudományi Egyetem könyvtári repozitori rendszerébe. Tudomásul veszem, hogy a megvédett és

- nem titkosított dolgozat a védést követően
- titkosításra engedélyezett dolgozat a benyújtásától számított 5 év eltelte után

nyilvánosan elérhető és kereshető lesz az Egyetem könyvtári repozitori rendszerében.

Kelt: 2023. év 11. hó 1. nap

Hallgató aláírása

MATE Szervezeti és Működési Szabályzat
III. Hallgatói Követelményrendszer
III.1. Tanulmányi és Vizsgaszabályzat
6.13. sz. függeléke: A MATE egységes szakdolgozat / diplomadolgozat / záródolgozat / portfólió készítési útmutatója
4.1. sz. melléklete: Konzulensi nyilatkozat

NYILATKOZAT

BASA MÁRTON (név) (hallgató Neptun azonosítója: CS1BR7) konzulenseként nyilatkozom arról, hogy a záródolgozatot/szakdolgozatot/diplomadolgozatot/portfóliót[1] áttekintettem, a hallgatót az irodalmi források korrekt kezelésének követelményeiről, jogi és etikai szabályairól tájékoztattam.

A záródolgozatot/szakdolgozatot/diplomadolgozatot/portfóliót a záróvizsgán történő védésre **javaslom / nem javaslom**[2].

A dolgozat állam- vagy szolgálati titkot tartalmaz: igen nem*[3]

Kelt: Gödöllő 2023 év 10 hó 31 nap

belső konzulens

[1] A megfelelő dolgozattípus meghagyása mellett a többi típus törlendő.
[2] A megfelelő aláhúzandó.
[3] A megfelelő aláhúzandó.