Funkcje chromatyny w transkrypcji, odpowiedzi na stres oraz rekombinacji mejotycznej u *Arabidopsis thaliana*

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Functions of chromatin in transcription, stress response
and meiotic recombination in *Arabidopsis thaliana*

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Funding

Profile of the PhD candidate

I graduated from high school in 2014 with major in Biology and Chemistry. I started my BSc. studies in Biotechnology in the same year at Adam Mickiewicz University in Poznań. In 2017 I obtained Bachelor's degree in the Department of Microbiology. My research then focused on determining the abundance of antibiotic resistance genes in water bodies in Poznań area. In the same year I enrolled for the Master in Biotechnology program at Adam Mickiewicz University. I have received the Best Student scholarship in 2018. During my Master studies I participated in Erasmus + program for 6 months at University of Jaén, Spain. After coming back from the scientific exchange, I joined Laboratory of Genome Biology to pursue Master Degree which I obtained with honors in 2019.

In 2019 I started PhD studies in the newly formed Doctoral School of Natural Sciences at Adam Mickiewicz University in Poznań under the supervision of Prof. Piotr Ziółkowski. The initial goal of my thesis was to find a functional relationship between transcription and occurrence of meiotic recombination. Additionally, I was interested in chromatin functions in regulation of stress gene expression. During my PhD studies, I co-authored 3 research articles. I am a first-joint author of one of those and a second author in two others. Together with my supervisor I published a review article. Additionally, I co-authored one handbook protocol. I was awarded START stipend funded by the Foundation for Polish Science and received AMU Foundation Scholarship for Best PhD Students.

I completed internship in Department of Molecular Genetics at Institute of Bioorganic Chemistry, PAS in Poznań. Moreover, I carried out RT-qPCR COVID-19 testing in the Coronavirus Laboratory at Medical University in Poznań during pandemic outbreak. I participated in international scientific conferences with oral and poster presentations. In September 2024 I co-organized Plant Meiosis Meeting in Poznań. Additionally, I took part in NGS data analysis training and EMBO PhD Course in Heidelberg.

Streszczenie

Stopień kondensacji chromatyny wpływa na wiele procesów biologicznych w skali komórkowej, takich jak inicjacja transkrypcji genów czy rekombinacja mejotyczna. Z drugiej strony, drobne zmiany molekularne zachodzące na poziomie nukleosomalnym mogą wpływać na takie czynniki jak reakcja na stres czy różnorodność genetyczna na poziomie organizmalnym i populacyjnym.

W pierwszej części pracy zbadałem funkcje i wpływ modyfikatorów chromatyny na ekspresję genów odpowiedzi na stres u rośliny modelowej *Arabidopsis thaliana*. Opisaliśmy acetylotransferazę NuA4, która odpowiada za acetylację histonu H4 oraz wariantu H2A.Z. Obecność NuA4 jest niezbędna do ekspresji genów związanych ze wzrostem i fotosyntezą. Z drugiej strony NuA4 stymuluje depozycję H2A.Z w genach związanych z reakcją na stres, co ostatecznie prowadzi do ich wyciszenia w fizjologicznych warunkach. Następnie zbadałem rolę enzymu deacetylazy histonowej HDA19, która może specyficznie usuwać grupy acetylowe z H2A.Z i H3. Równowaga pomiędzy acetylacją i deacetylacją histonów tworzy zatem mechanizm zapobiegający spontanicznej ekspresji genów stresowych.

W dalszej części zbadałem możliwość modyfikowania chromatyny poprzez naprowadzanie systemu opartego na nieaktywnej katalitycznie endonukleazie dCas9. Wyniki wskazują na to, że lokalna rekrutacja peptydu VP64, prowadzi zarówno do zwiększonej ekspresji wybranego genu oraz do lokalnej reorganizacji w strukturze chromatyny, która może wpływać na zachodzenie rekombinacji mejotycznej (crossing-over, CO) w wybranym locus.

W drugiej części dysertacji przedstawiłem metody bioinformatyczne wykorzystywane w analizie danych NGS, ze szczególnym uwzględnieniem wykrywania zdarzeń crossing-over. Przygotowałem prosty i skuteczny sposób określania miejsca zachodzenia crossing-over w skali hotspotu rekombinacyjnego, który wykorzystywany jest w metodzie *seed-typing*. Dodatkowo, zaprojektowałem narzędzie internetowe oparte na środowisku RShiny, które pozwala na szybkie porównanie zestawów danych, pochodzących z różnych opublikowanych do tej pory eksperymentów typu genotyping-by-sequencing (GBS).

Podsumowując, przedstawiłem różne aspekty mojej działalności badawczej, począwszy od badań nad rolą chromatyny w ekspresji genów stresowych i rekombinacji mejotycznej, a skończywszy na podejściu obliczeniowym w analizie danych biologicznych.

Słowa kluczowe: chromatyna, mejoza, crossover, odpowiedź na stres, ekspresja genów, bioinformatyka, GBS, Arabidopsis

Abstract

Chromatin composition and accessibility affect multiple biological processes on cellular scale, such as initiation of gene transcription or meiotic recombination. In turn, those molecular changes, affect genetic diversity and stress-response on the populational and organismal levels.

In the first part of the thesis, I have investigated functions of chromatin modifications on expression of stress-response genes in model plant species *Arabidopsis thaliana*. We thoroughly characterized histone variant H2A.Z acetyltransferase NuA4 that is necessary for expression of genes related to growth and development. On the other hand, NuA4 stimulates deposition of H2A.Z into gene-bodies of stress-responsive genes, which ultimately leads to their silencing in non-inductive conditions. Later, I investigated roles of histone deacetylase enzyme HDA19, that can specifically remove acetyl groups from H2A.Z and H3 at responsive genes. Interplay between histone acetylation and deacetylation forms equilibrium preventing spurious expression of stress genes.

In the subsequent section I explored possible roles of chromatin modifications with dCas9-based tools. I provided a set of evidence that local recruitment of expression inducer peptide VP64, leads not only to increased transcription of the targeted gene but also to modifications in chromatin which can affect meiotic crossover formation in the targeted locus.

In the second part of the thesis, I developed bioinformatic pipelines used in New Generation Sequencing (NGS) data analysis with emphasis on crossover detection in *Arabidopsis*. I developed a code that provides a simple and effective way to determine crossover site in NGS data generated via seed-typing. Seed-typing is a method for investigating meiotic recombination at the hotspot scale. I also designed web-based tool that allows easy browsing of genotyping-by-sequencing datasets coming from different mutant backgrounds.

To sum up, I demonstrated various aspects of my PhD research activities, ranging from studies on chromatin roles in the regulation of gene expression and meiotic recombination to development of computational approaches in biological data analysis.

Published works included in the dissertation

Part I:

1. Bieluszewski, T., Szymanska-Lejman, M., **Dziegielewski, W**., Zhu, L., & Ziolkowski, P. A. "Efficient Generation of CRISPR/Cas9-Based Mutants Supported by Fluorescent Seed Selection in Different Arabidopsis Accessions" *Methods Mol Biol* 2484, 161–182 (2022).
   https://link.springer.com/protocol/10.1007/978-1-0716-2253-7_13

2. Bieluszewski, T.*, Sura, W.*, **Dziegielewski, W**.*, Bieluszewska, A., Lachance, C., Kabza, M., Szymanska-Lejman, M., Abram, M., Wlodzimierz, P., De Winne, N., De Jaeger, G., Sadowski, J., Côté, J., & Ziolkowski, P. A. "NuA4 and H2A.Z control environmental responses and autotrophic growth in Arabidopsis" *Nat Commun* 13, 277 (2022) ***Co-first author**.
   https://www.nature.com/articles/s41467-021-27882-5

3. **Dziegielewski, W**., & Ziolkowski, P. A. "License to Regulate: Noncoding RNA Special Agents in Plant Meiosis and Reproduction" *Front Plant Sci* 12, 662185 (2021).
   https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2021.662185/full

Part II:

1. Szymanska-Lejman, M., **Dziegielewski, W**., Dluzewska, J., Kbiri, N., Bieluszewska, A., Poethig, R.S. & Ziolkowski, P.A. "The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids" *Nat Commun* 14, 33 (2023).
   https://www.nature.com/articles/s41467-022-35722-3

2. Dluzewska, J., **Dziegielewski, W**., Szymanska-Lejman, M., Gazecka, M., Henderson I.R., Higgins, J.D., Ziolkowski P.A. "MSH2 stimulates interfering and inhibits non-interfering crossovers in response to genetic polymorphism" *Nat Commun* 14, 6716 (2023).
   https://www.nature.com/articles/s41467-023-42511-z

List of abbreviations

AMOR	Arabidopsis Maps of Recombination
bp	base pair
BRB-seq	Bulk RNA Barcoding and sequencing
BT	BowTie interval
ChP	Chili Pepper interval
cM	centimorgan
CO	crossover
Col	Columbia (Arabidopsis accession)
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats, CRISPR-associated protein 9
dCas9	deadCas9
dHJ	double Holliday junction
D-loop	displacement loop
DMC1	disrupted meiotic cDNA
DSB	DNA double-strand break
dsRED	Discosoma red fluorescent protein
eGFP	enhanced green fluorescent protein
ESIL	Extremely Short Interval Line
FANCM	FA complementation group M
GBS	genotyping-by-sequencing
GLK	Golden2-Like
gRNA	guide RNA
HAT	histone acetyltransferase
HDA19	histone deacetylase 19
H3K9ac	histone H3K9 acetylation
H2A.Zac	acetylated histone variant H2A.Z
H3K4me3	histone 3 lysine 4 tri-methylation
H3K27me3	histone 3 lysine 27 tri-methylation

HEI10	homolog of human HEI10 (enhancer of cell invasion no.10)
HDAC	histone deacetylase
HR	homologous recombination
kb	kilobase
L*er*	Landsberg *erecta* (Arabidopsis accession)
lncRNA	long non-coding RNA
Mb	megabase
miRNA	microRNA
MMR	mismatch repair system
MSH2	MutS homolog 2
NCO	noncrossover
ncRNA	non-coding RNA
NHEJ	non-homologous end joining
NuA4	Nucleosomal Acetyltransferase 4
PRDM9	PR/SET Domain 9
RECQ4	RECQ helicase
RF	recombination frequency
SDSA	synthesis-dependent strand annealing
siRNA	short-interfering RNA
SNI1	suppressor of NPR1, inducible
SNP	single nucleotide polymorphism
SPO11	sporulation-specific protein 11
TF	transcription factor
TIGER	Trained Individual GenomE Reconstruction
TSS	transcription start site
VP64	tetrameric Virulent Protein 16 from *Herpes simplex*
ZMM	class I crossover pathway, name comes from the first letters of the proteins required for this process in yeast: ZIP (ZIP1, ZIP2, ZIP3 and ZIP4), MER3, and MSH (MSH4 and MSH5)

Introduction

Chromatin composition and regulation

Chromatin is a complex structure organized of DNA, RNA and proteins. Its repetitive and functional unit is called a nucleosome, consisting of 146 DNA base pairs wrapped around 8 histone proteins. Four pairs of canonical histone proteins H2A, H2B, H3 and H4 form a core octamer, to which DNA is bound by electrostatic bonds (Hübner et al., 2013). Negatively charged DNA is tightly attached to histone proteins that present positive charge on their N-terminal lysine or arginine residues. Nucleosome occupancy limits activity of DNA-acting proteins, however the levels of chromatin accessibility for different transcription factors (TFs) or other proteins can be regulated by various factors that can transiently neutralize the charge on histone proteins (Lee et al., 2004; Zhang et al., 2007). Repeating units of nucleosomes form a 10 nm fibre structure, referred to as "beads on a string" (Baldi et al., 2020). Individual nucleosomes are separated by 20-90 DNA base pairs of linker DNA, stabilized by linker histone H1 located outside of the nucleosome core. Such a structure is called chromatosome. Presence of linker histone promotes formation of higher order structures, such as the 30 nm fibre observed *in vitro* (Routh et al., 2008). The 30 nm fibre models assume either *zigzag* or *solenoid* organization but it is possible that both these form coexist, making the whole structure highly heterogeneous (Razin & Gavrilov, 2014). Nevertheless, the 30 nm fibre structure has not been observed *in vivo* and its presence in the living cells has been recently questioned (Maeshima et al., 2019). Nucleosomes are further condensed into nucleus or individual chromosomes in the dividing cells (Luger & Dechassa, 2012; Maeshima et al., 2019).

Advancements in microscopy and novel methods aimed at defining the 3D composition of chromatin, such as Hi-C led to re-organizing the genome into higher-order structures, defined by their functional domains (Misteli, 2020). First, nucleosomes can be condensed to form chromatin loops and stabilized by the presence of transcription factors. Chromatin loops play a major role in regulation of transcription as they can influence gene expression in the regions adjacent to a loop, but also localised several thousand of base pairs away on the same or different chromosome (Vermunt et al., 2019). Another types of structural chromatin organization are Topologically Associated

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Domains (TADs). They span over thousands (kb) to millions of base pairs (Mb) and facilitate interactions of regulatory elements with genes located within their borders (McArthur & Capra, 2021). Adjacent TADs are separated and isolated from each other by structural proteins such as cohesin or CTCF (CCCTC-binding factor) but also through chromatin loops (Pei et al., 2021). Although in *Arabidopsis* no TADs have been identified, genomes of rice, tomato or maize show existence of those structures (Dong et al., 2017). Plant TADs differ from their animal counterparts, as they lack CTCF protein while chromatin loops can extend through the structure borders and regulate expression of genes found in the neighbouring domains (Dong et al., 2017; Pei et al., 2021).

A/B compartments built of several Mb are defined by the type of chromatin that composes those regions. "A" compartment consists of euchromatin - transcriptionally active, enriched in gene-coding sequences and other regulatory elements with more accessible structure. On the other hand, "B" compartment comprises heterochromatin and is more condensed (Dong et al., 2017). In *Arabidopsis* euchromatin occupies mostly interstitial parts of the chromosome arms, while heterochromatin is found at pericentromeric and telomeric regions, however in species with large genomes, heterochromatin can occupy larger regions (Janssen et al., 2018; Lorković & Berger, 2017). On top of chromatin loops, TADs and A/B compartments, chromosome territories (CTs) are formed. During interphase, chromosomes occupy defined spaces in the nucleus. The way of CT organization can be specific to a given species (Doğan & Liu, 2018).

Histone post-translational modifications

High evolutionary sequence and structure conservation of canonical histone proteins throughout eukaryotes reflect their importance for genome maintenance and stability (Talbert & Henikoff, 2010). Different amino acid residues on histone tails can be targets of epigenetic post-translational modifications (PTMs), such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation or ADP-ribosylation. Presence of such chemical changes, influences the chromatin accessibility, thus altering the important molecular processes, such as DNA repair, gene expression, replication, recombination or cell death (Greer & Shi, 2012; Jenuwein & Allis, 2001; Zhang et al., 2007).

One of the reversible dynamic histone PTMs is histone acetylation. The acetyl groups coming from acetyl-CoA are deposited at lysine residues on histone tails by different histone acetyltransferase (HATs) complexes. There are 4 main HAT families in *A. thaliana*: GNATs (GCN5-related N-terminal acetyltransferases), MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60), CBP (p300/CREB-binding protein related) and transcription initiation factors TAF$_{II}$250-related (Pandey et al., 2002; Roth, 2001). The general role of histone acetylation is associated with transcriptional activation. Addition of acetyl group to lysine residues, neutralizes the positive charge of the histone tail. In effect, such acetylated histone has a lower affinity to negatively charged DNA, which allows binding of other DNA-acting proteins, like TFs. Apart from the direct influence of HATs on gene expression, the acetylated lysine groups can serve as signalling hallmarks, read by bromodomain-containing chromatin remodelling complexes, such as SWI/SNF (Jarończyk et al., 2021; Yu et al., 2021). On the other hand, histone deacetylases (HDACs) counteract HAT activity and remove acetyl groups from Lys residues, leading to a stronger interaction between DNA and histones, ultimately leading to transcriptional silencing (Figure 1). There are 18 putative histone deacetylases in *A. thaliana*, grouped in 3 families, called RPD-3/HDA1, sirtuins and plant specific HD-tuins (Alinsug et al., 2009). Balance between acetylation and deacetylation of histone proteins regulates multiple cellular and biological processes in plants, such as photosynthesis, transition from the vegetative to generative growth and stress response (Bieluszewski et al., 2022; Shvedunova & Akhtar, 2022; Tian et al., 2019).
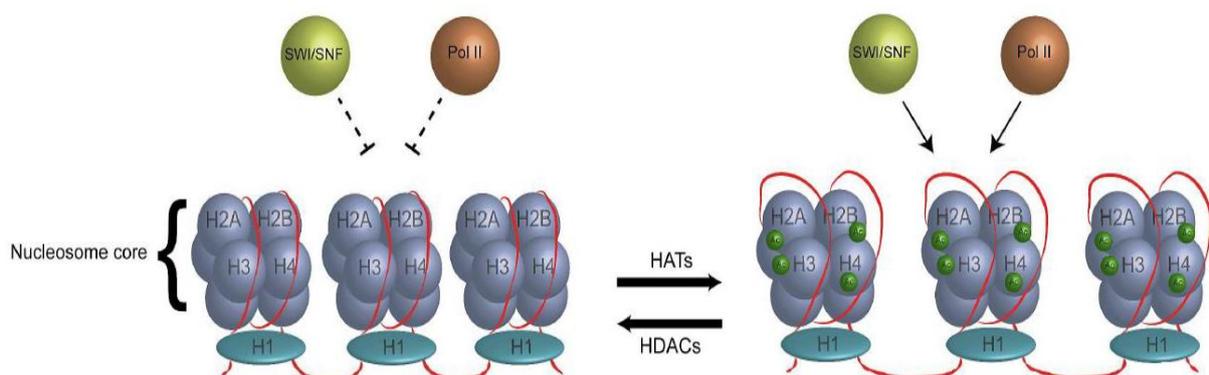

Figure 1. Schematic representation of histone acetylation dynamics.

Histones forming the nucleosome core can be replaced by their non-canonical variants. These are isoforms of histone proteins that gained functionality in the process

of convergent evolution (Talbert & Henikoff, 2017). Variants of H2A family in *Arabidopsis* include H2A.W, H2A.X and H2A.Z. H2A.W was reported to be associated with heterochromatin and silencing of the transposon genes (Yelagandula et al., 2014). H2A.X is deposited near DNA damage sites, is phosphorylated by kinases ATM and ATR and then acts in recruitment of DNA repair machinery (Donà & Scheid, 2015). H2A.Z is deposited at transcription start sites (TSS) of actively transcribed genes, but can be also enriched over gene bodies of transcriptionally repressed genes (Coleman-Derr & Zilberman, 2012). A multicomponent complex SWR1-C is responsible for this process in *Arabidopsis*, however its efficient deposition depends on HAT histone acetyltransferase complex NuA4 (Bieluszewski et al., 2022; Deal et al., 2005; Gómez-Zambrano et al., 2018).

Likewise their canonical counterparts, histone variants also undergo PTMs. Modifications of H2A.Z, including acetylation, influence the dual nature of this histone variant, which maintains balance between activation of transcription and repression of stress-related genes under physiological conditions (Sura et al., 2017).

NuA4 histone acetyltransferase

NuA4 is a HAT belonging to MYST family, firstly described in yeast *Saccharomyces cerevisiae*, as a main complex catalysing acetylation of lysine residues on histones H4, H2A and its non-canonical variant Htz1 (yeast H2A.Z homolog) (Allard et al., 1999; Keogh et al., 2006). This complex is composed of 13 smaller subunits, which are often shared with other multi-component complexes, such as SAGA or SWR1. Subunits forming NuA4 in yeasts are: Act1 (Actin 1), Arp4 (Actin Related Protein 4), Eaf1/3/5/6/7 (ESA-1 Associated Factor), Epl1 (Enhancer of Polycomb-Like 1), Esa1 (Essential SAS2-related Acetyltransferase 1), Swc4 (SWR1-Complex Protein), Tra1 (Transcription-associated protein 1), Yaf9 (Yeast AF-9) and Yng2 (Yeast Inhibitor of Growth 2) (Allard et al., 1999; Wang et al., 2018). Additionally, two submodules of NuA4 exist *in vivo*: piccolo-NuA4, composed of Esa1, Epl1, Yng2 and Eaf6 that is able to perform non-directed acetylation and the TINTIN complex composed of Eaf5/7/3 subunits. Interestingly, NuA4 in humans has probably fused together with SWR1 complex into one, to perform histone acetylation and variant deposition more efficiently at the same time (Perez-Perri et al., 2016; Rajagopalan et al., 2017).

Studies in yeast have shown that one of the main functions of NuA4 is transcriptional regulation. The catalytic module piccolo-NuA4 acetylates histone H4, leading to higher chromatin accessibility, affecting the rate of TFs binding (Utley et al., 1998). Other studies have suggested that the positive effect of piccolo-NuA4 on gene expression may be also associated with later steps of transcription as NuA4 cooperates with GCN5, acetyltransferase domain of SAGA, in stimulating the rate of polymerase elongation (Ginsburg et al., 2009). Another important role of NuA4 complex is its involvement in DNA double strand breaks (DSBs) repair by HR (homologous recombination) in yeast. After the lesion, the MRX (Mre11-Rad50-Xrs2) complex is recruited to DSBs site, which later recruits Mec1 kinase that phosphorylates H2A.X (γH2A.X). Spreading of γH2A.X recruits NuA4 and SAGA that remodel chromatin on both sides of the DSB during end resection. NuA4 acetylates Replication Protein A (RPA), that further facilitates HR processes, such as Rad51 loading on DNA (Cheng et al., 2018, 2021). Functional NuA4 is necessary for efficient process of H2A.Z deposition in yeasts and mammals (Altaf et al., 2010; Choi et al., 2009).

The plant NuA4 complex has been poorly understood until recent years. Esa1 homologues have been identified as HAM1 and HAM2 required for acetylation of histone H4 (H4ac), crucial for gametogenesis and flowering time regulation in *A. thaliana* (Latrasse et al., 2008; Xiao et al., 2013). Other subunit, YAF9A has been described as part of MLK4 kinase complex, required for H2A.Z loading at genes controlling flowering time, such as *GIGANTEA* (Su et al., 2017). Additionally, other individual NuA4 complex homologous proteins have been discovered in *Arabidopsis*, but none of these studies successfully captured the existence and function of holo-NuA4 complex (Bieluszewski et al., 2015; Tan et al., 2018; Xu et al., 2014). Recent studies have demonstrated the composition and roles of *Arabidopsis* NuA4. It was shown that expression of genes required for development and photosynthesis directly depends on H4ac and H2A.Zac catalysed by NuA4. Similarly to yeasts and animals, activity of plant NuA4 is also indispensable for H2A.Z loading (Barrero-Gil et al., 2022; Bieluszewski et al., 2022a; Zhou et al., 2022).

Stress response in plants

The challenging environmental conditions pose threat to growth and development of sessile plants that are unable to escape from the unfavourable environment or pathogens. Instead, plants have employed various sensing, signalling and defence mechanisms used for managing biotic and abiotic stresses. The strategies of response include direct regulation of gene expression, alternative transcript processing or post-translational modifications.

Perturbations in the cell components, caused by abiotic factors can serve as molecular sensing mechanisms. Osmotic stress can be sensed by changes in the turgor pressure, plasma membrane curvature or receptors localised in cytoplasm. OSCA1 has been described in *Arabidopsis* as osmosensic calcium-permeable channel, that transduces Ca^{2+} in response to hyperosmolarity (Yuan et al., 2014). Temperature changes can be recognized by various transcription factors such as TWA1, C-REPEAT BINDING FACTOR/DEHYDRATION RESPONSIVE ELEMENT (DRE)-BINDING1 (CBF/DREB1) or HEAT SHOCK FACTOR A1 (HsfA1) that can directly influence expression of responsive genes (Bohn et al., 2024; Lohmann et al., 2004; Yamaguchi-Shinozaki & Shinozaki, 1994). Moreover, phytochromes have been shown to recognize both light and high temperature signals (Jung et al., 2016; Legris et al., 2016).

Receptor-like kinases (RLKs) are transmembrane proteins that act in communication between cells and the environment (Walker & Zhang, 1990). Based on their amino acid composition, they were classified into more than 21 families in *Arabidopsis*, however the most numerous subfamily is called LRR (leucine rich repeat)-RLK (Gou et al., 2010). The LRR-RLKs are found in many plant species like *Oryza sativa*, *Solanum lycopersicum* or *Zea mays* (Song et al., 2015; Wei et al., 2015). They act as surface receptors, recognizing various molecules outside of the cell and starting the downstream phosphorylation cascades, leading to modification of cellular activity.

After sensing the stress, signal is propagated in the cell through second messengers, such as calcium, reactive oxygen species (ROS) or nitric oxide (NO). Ca^{2+} can be sensed by calcium-binding proteins, leading to activation of protein kinases. ROS, produced by NADPH oxidase RbohD, is likely detected by HPCA1 and can stimulate further long-distance responses to diverse stimuli (Miller et al., 2009; Wu et al., 2020).

Under unfavourable conditions, high amounts of specific stress-related transcripts are produced. A properly working RNA processing factors are necessary for transcript maturation, which is reflected by upregulation of transcriptional machinery elements during stress (Lu et al., 2020; Wang et al., 2020). Alternative splicing of signal transducing phosphatases PP2C pre-mRNAs was observed in ABA-stressed plants (Wang et al., 2015).

Plants apart from abiotic cues are also exposed to infection by various parasites, pests and pathogens, causing biotic stress. The adverse effects caused by pathogen infection include necrosis, wilting, stunting, chlorosis or tumour growth (Osman et al., 2020; Pallas & García, 2011; Sobiczewski et al., 2017). The presence of pathogen is detected by pattern recognition receptors (PRRs), that are able to detect the microbial structures. PRRs include previously mentioned LRR-RLKs, able to recognize bacterial flagellin or fungal chitin-oligomers (Couto & Zipfel, 2016; Yu et al., 2017). Subsequent stages after pathogen recognition include signal transduction based on ROS spikes and Ca^{2+} bursts that can activate calcium dependent kinases and mitogen-activated protein kinase (MAPK) cascade. Interestingly, pathogens avoid recognition through PRRs by constant evolving of flagellin or different effector proteins (Lopez et al., 2019; Malvino et al., 2022; Zembek et al., 2018).

Post-translational histone modifications at promoter sequences, resulting from stress signalling, influence expression of stress-responsive genes. Chromatin modifications, such as deposition of acetyl- or methyl- groups at specific histone lysine residues of responsive genes, have been shown to trigger expression under stress conditions (Sani et al., 2013). Hyperacetylation of H3K9 and H3K14, mediated by GCN5 acetyltransferase complex is associated with higher expression of stress genes under high salt conditions in *Arabidopsis* (Zheng et al., 2019). Repressive mechanisms are often based on RNA-directed DNA methylation (RdDM) pathway, connected with further establishment of heterochromatic histone marks such as H3K9me2 or H3K27me3 (Carter et al., 2018). DNA can also undergo demethylation process at promoters of cold and drought response genes in response to stimuli, reported in different plant species (Conde et al., 2017; Zhang et al., 2020).

One of the well-studied chromatin modifications connected to stress response is deposition of histone variant H2A.Z. H2A.Z is usually deposited at +1 nucleosome, which is the nucleosome following the transcription start site (TSS). Alternatively, it can

be deposited in the latter nucleosomes forming the gene body. Role of H2A.Z is highly context and location dependent, as H2A.Z can have either a promoting or repressive role in gene expression. Model proposed in *Drosophila*, assumed that the presence of acetylated H2A.Z (H2A.Zac) at +1 nucleosome could decrease the energy used by RNA Polymerase II (RNAPII) to overcome the first nucleosomal barrier and initiate transcription process (Weber et al., 2014). When H2A.Z is deposited in the gene body region, it exerts inhibitory effect on gene expression, probably leading to polymerase stalling. Genes involved in stress-response show higher levels of gene body H2A.Z, which represses their expression in physiological conditions (Coleman-Derr & Zilberman, 2012). Induction of stress, leads to eviction of H2A.Z from chromatin which allows upregulation of responsive genes and adaptation to a new environmental conditions (Sura et al., 2017).

The main complex responsible for H2A.Z eviction from nucleosome in *Arabidopsis* is INO80, however some additional factors might be involved in this process (Xue et al., 2021). The eviction rate scales with temperature increase, as plants grown at 27°C have lower H2A.Z occupancy than those grown at lower temperatures (Kumar & Wigge, 2010). Recent reports demonstrated that H2A.Z eviction is facilitated by HDA9 and PWR proteins (Tasset et al., 2018). Investigating what other HDAC proteins are involved in processes of H2A.Z deposition and deacetylation, could lead to discovery of novel epigenetic control mechanism over responsive genes in plants, as some studies indicate that the PTMs of H2A.Z and not the presence of H2A.Z itself, can be crucial factors influencing the gene expression (Gómez-Zambrano et al., 2019; Valdes-Mora et al., 2012).

Role of histone deacetylases in stress response

There are 18 HDACs in *Arabidopsis*, grouped in 3 subfamilies (Alinsug et al., 2009). 12 of them belong to Zn^{2+} Reduced Potassium Dependency3 (RPD3/HDA1) subfamily, divided into three classes (I, II, IV). Plant specific HD-tuins consist of 4 histone deacetylases. 2 sirtuins belong to SIR2 subfamily (Ma et al., 2013). High number of histone deacetylases found in *Arabidopsis* point out to their functional redundancy but also to antagonistic roles in stress response (Table 1). Multiple studies have indicated that HDACs can influence various biological processes (Luo et al., 2012; Morończyk

et al., 2022; Wu et al., 2008). For example, Class I mutants such as *hda19* and *hda9* are more tolerant to salt stress, but quadruple *hda5/14/15/18* are hypersensitive to salt stress (Ueda et al., 2017; Zheng et al., 2020). Moreover, Class I *hda6* mutants display tolerance to drought in contrast to *hda15*, showing drought hypersensitivity (Kim et al., 2017; Lee & Seo, 2019). In case of temperature response, *hda9* and *hda19* display warm-insensitive phenotype, oppositely to *hda15* mutants (Shen et al., 2019). Plant specific HD-tuins are involved in drought and heat stress responses (Buszewicz et al., 2016; Han et al., 2016). Additionally, expression of heat, drought or osmotic resistance genes is affected in different *hda* mutants (Han et al., 2016; Shen et al., 2019; van der Woude et al., 2019).

Apart from histone tails, lysine residues can be acetylated at a large number of proteins. Several HDACs can deacetylate non-histone substrates thus affecting transcription, translation and metabolism processes.

Transcription factors such as BIN2 (repressor of brassinosteroid signalling) can undergo deacetylation catalysed by HDA6, which represses its kinase activity (Hao et al., 2016). Deacetylation of WRKY53, a general stress-responsive TF is mediated by HDA9 (Chen et al., 2016; Zheng et al., 2020). The specific targeting of HDACs to TFs in stress conditions remains unclear. Metabolic enzymes involved in photosynthesis can be deacetylated by class II HDA14 which presence was detected in chloroplasts (Hartl et al., 2017). Additionally, HDA14 can catalyse acetyl group removal from α-tubulin (Tran et al., 2012). Histone deacetylase belonging to sirtuin subfamily, SRT2 acts on inner mitochondrial membrane proteins and influence metabolic processes (Konig et al., 2014). Recent report has shown ribosomal proteins and translation factors might be regulated by histone deacetylase activity in rice (Xu et al., 2021). Interestingly, emerging roles of HDACs have been recently reported in the context of H2A.Z eviction or deposition under stress conditions (Tasset et al., 2018; van der Woude et al., 2019).

Table 1. Roles and classification of histone deacetylases in *Arabidopsis thaliana*.

Family	Class	HDA	Role
RPD-3/HDA1	Class I	HDA6	• Control of DNA methylation and chromatin silencing (Hristova et al., 2015; Yang et al., 2020) • Interacts with MET1 methyltransferase and acts as global epigenetic regulator (Vincent et al., 2022; Yu et al., 2011)
		HDA7	• Ensures proper female gametophyte development but is catalytically inactive (Cigliano et al., 2013; Saharan et al., 2024)
		HDA9	• Stress and flowering regulator (Nguyen et al., 2023; Zheng et al., 2020) • Responsible for deacetylation of H3/H4 (Kim et al., 2016; Mayer et al., 2019) • Takes part in H2A.Z eviction from chromatin in high temperature (van der Woude et al., 2019)
		HDA10	• Pseudogenes originating from partial duplication of HDA9 (Hartl et al., 2017)
		HDA17	
		HDA19	• Controls seed maturation, embryogenesis, flowering and ovule development (Gao et al., 2015; Manrique et al., 2024; Ning et al., 2019) • Regulates expression of stress-response genes (Shen et al., 2019)
	Class II	HDA5	• Tandemly duplicated genes (Ueda et al., 2017)
		HDA18	• Potential role in deacetylation of non-histone proteins (Alinsug et al., 2012)
		HDA8	• Found in chloroplasts, possible roles in deacetylation of non-histone proteins (Alinsug et al., 2012; Tran et al., 2012)
		HDA14	
		HDA15	• Interacts with PIF3 and controls biosynthesis of chlorophyll and expression of stress-response genes (Liu et al., 2013; Shen et al., 2019; Tu et al., 2022)
	Class IV	HDA2	• Unknown
Sirtuins		SRT1	• Regulate glycolysis and energy metabolism (Bruscalupi et al., 2023; Konig et al., 2014; Zhang et al., 2018)
		SRT2	
HD-tuins		HDT1	• Control leaf polarity and possibly stress response (Luo et al., 2022; Pontes et al., 2007)
		HDT2	
		HDT3	• Regulates stress-response genes (Buszewicz et al., 2016; Luo et al., 2022)
		HDT4	• Negative regulator of flowering (Farhi et al., 2017)

Regulation of meiotic recombination at the chromatin level

Apart from transcription, chromatin regulates multiple DNA-associated biological processes. One of those is meiotic recombination occurring during prophase of meiosis.

Meiosis is a specialized and conserved type of cell division found in sexually reproducing organisms resulting in production of four haploid cells from one parental diploid cell. There are four stages in the first meiotic division (meiosis I): prophase I, metaphase I, anaphase I and telophase I. Prophase I can be further divided into four steps: leptotene, zygotene, pachytene and diplotene. During pachytene, homologous chromosomes pair and reciprocally exchange DNA fragments. These events are called crossovers (COs) and are important sources of genetic diversity among populations, alongside random mating and random chromosome assortment during later stages of meiotic division (Jones & Franklin, 2006). Moreover, CO occurrence assures proper segregation of chromosomes in anaphase I (Shinohara et al., 2008). In metaphase I, homologous chromosomes form a metaphasal plate and during anaphase I, they are separated into opposite poles of cell, giving rise to two newly forming haploid cells. Meiosis II also consists of four stages and its progress is similar to mitotic division. During anaphase II, sister chromatids are separated that leads to production of four haploid gametes (animals) or spores (plants and fungi).

Meiotic recombination is always initiated by DSB, catalysed by conserved endonuclease SPO11 dimer (Keeney et al., 1997). After DSB initiation, the 5' ends of the DNA are resected, resulting in free 3'-ssDNA (single-stranded DNA) ends. Recombinases RAD51 and DMC1 are loaded on the DNA strands and mediate inter-homologue invasion and generation of a displacement loop (D-loop). A fraction of formed D-loops enter the second-end capture and double Holiday junctions are generated. These structures are further processed mainly (80-90%) by ZMM proteins and resolved as Class I CO (Mercier et al., 2015; Ziolkowski, 2023). Around 150-250 DSBs are formed in the cell per meiosis in *Arabidopsis*, however only about 5% of them will be resolved as COs, usually leading to 8-10 COs per cell. In *Arabidopsis* male sex-cells display slightly higher crossover numbers than the female cells (Drouaud et al., 2007; Salomé et al., 2012).

Majority of the DSBs are repaired through NCO pathway (non-crossover pathway, based on nonreciprocal exchange of one DNA sequence). In this pathway DNA helicases such as FANCM or RECQ4A/B disassemble intermediate joint-molecules and lead to a SDSA process (synthesis-dependent strand annealing). In this process, sequence flanking the DSB is replaced with an exact copy from homologous DNA template (Fig. 2) (Crismani et al., 2012; Girard et al., 2015; Higgins et al., 2011).

Meiotic DSBs usually happen in non-random chromatin accessible regions, known as hotspots (Tock & Henderson, 2018). Their distribution across the genome and pathway of DSB repair, have direct influence on resulting haplotype in individual forming gametes, as well as consequences in genetic diversity of natural populations (Cooper et al., 2016; Dluzewska et al., 2018).

Most of the forming CO events (85-90%) belong to Class I, mediated by ZMM proteins (coming from names of yeast proteins Zip1-4, Mer3, Msh4-5). In *Arabidopsis*, ZMM proteins have been identified as SHOC1, HEI10, ZIP4, PTD, MER3, MSH4 and MSH5. SHOC1 and PTD form a heterodimer that stabilizes D-loops and dHJs. HEI10 (functional homolog of yeast Zip3) is an E3 ubiquitin ligase that acts as a dosage dependent regulator of CO formation that establishes chromosomal foci, marking the sites of crossovers (Morgan et al., 2021; Ziolkowski et al., 2017). ZIP4 is thought to form a connection between crossover formation and synaptonemal complex assembly (Pyatnitskaya et al., 2022). MER3 acts as DNA helicase necessary for the second end capture (Chen et al., 2005; Mercier et al., 2005). MSH4 and MSH5 form a heteroduplex that recognizes and stabilizes the formation of a double-Holiday junction (Higgins et al., 2008).

Interestingly, Class I COs are located on chromosome further apart from each other, than it would be expected by chance. The presence of one Class I crossover strongly inhibits the occurrence of another one in a close proximity. This phenomenon of CO interference, has been observed for the first time in *Drosophila* (Sturtevant, 1915). For many years, it has been proposed that mechanical tensions that propagate from the Class I CO site obstruct formation of another crossover (Kleckner et al., 2004). Yet more recently, a HEI10-coarsening model emerged that might be more appropriate for description of interference phenomenon, at least in plants (Morgan et al., 2021). Minority of crossover intermediates are separated by interference-insensitive Class II helicases (Berchowitz et al., 2007).
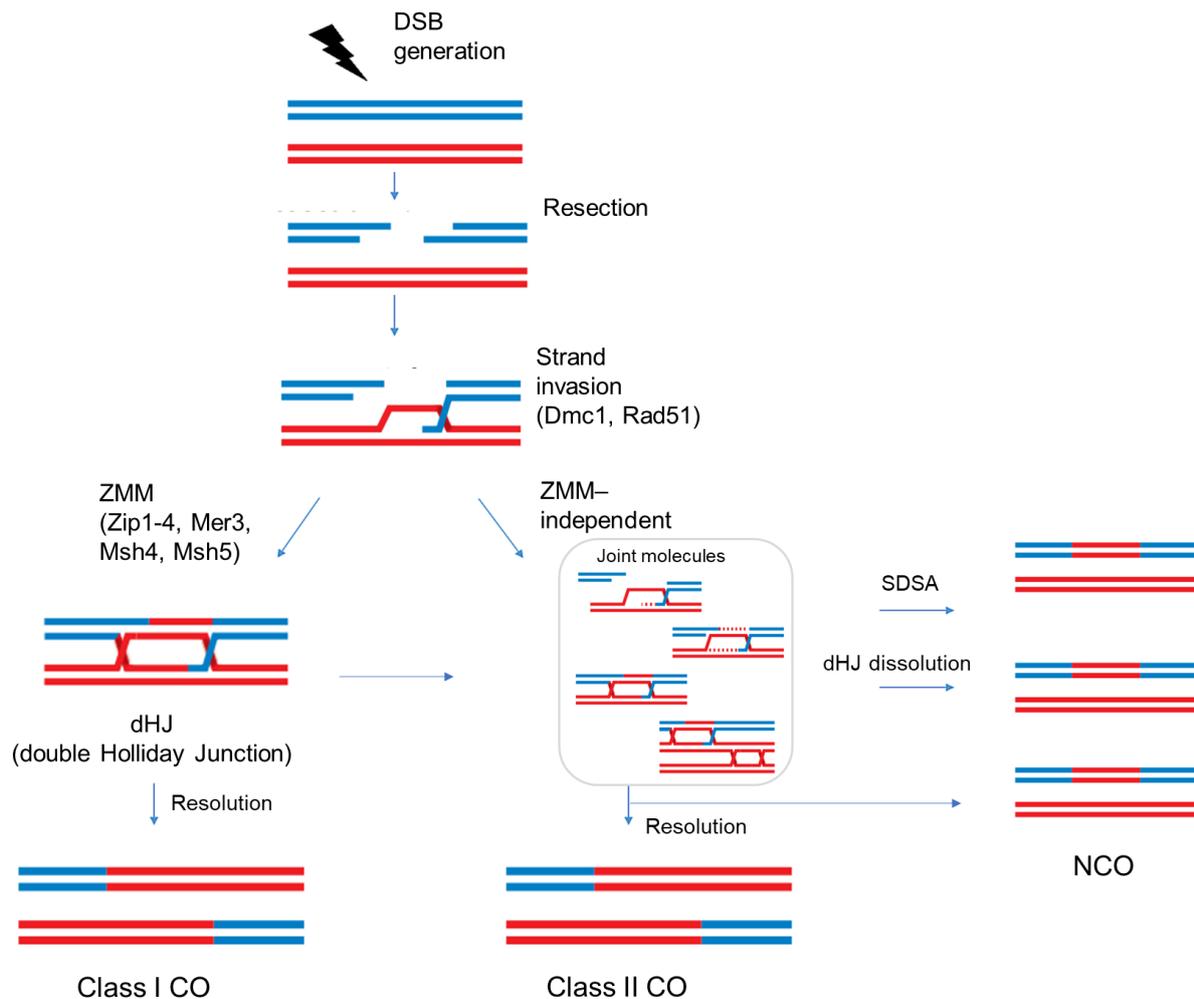
Figure 2. Biogenesis of crossover pathways. Based on Mercier et al., 2015.

Epigenetic modifications of chromatin affect the crossover patterning among different organisms. In all studied eukaryotes, COs overlap with regions of accessible chromatin (Choi et al., 2018; Fowler et al., 2014; Simorowski et al., 2018). One of the prominent proteins facilitating CO formation in most of the mammals is PRDM9 histone methyltransferase. It binds DNA through zinc-finger domain and catalyses H3K4 and H3K36 methylation, that recruits DSB machinery, including SPO11 (Diagouraga et al., 2018). Despite its importance in CO formation, evolutionary history shows multiple losses of *PRDM9* gene in vertebrates, suggesting its partial dispensability for the process (Cavassim et al., 2022). Indeed, species lacking PRDM9, such as plants or yeasts still form crossovers at sites of H3K4me3, suggesting the role of this epigenetic mark in determination of DSB repair pathway (Borde et al., 2009; Choi et al., 2018). In *Arabidopsis*, COs tend to colocalize to gene promoters and nucleosomes containing H2A.Z histone variant (Choi et al., 2013). Additionally, cytosine methylation forms

another layer of CO regulation. Plants lacking CG, CHG or CHH DNA display increased crossover rates in euchromatic and pericentromeric regions (Underwood et al., 2018; Yelina et al., 2015). It is worth noting that meiosis can be also controlled by different non-coding RNA (ncRNA) molecules, influencing transcription control, chromatin condensation, DNA methylation or development of reproductive organs (Dziegielewski & Ziolkowski, 2021).

Aims and objectives

Although histone acetylation is playing a major role in transcriptional regulation, relationship between H2A.Z acetylation, deposition and its dual role in gene expression remains unclear. First aim of this thesis was to untangle the role of H2A.Z deacetylation in control of stress-response genes repression.

Hypothesis 1: Histone deacetylates remove acetyl groups from H2A.Z, after its deposition in acetylated form at gene-bodies, leading to repression of stress-response genes.

Chromatin modifications found at transcriptionally active promoters are similar to those connected to sites with higher meiotic recombination frequency. The second aim of the thesis was to explore relationship between transcription and crossover formation, by employing dCas9-based system, aimed to induce expression at recombination hotspot.

Hypothesis 2: Ectopically increased gene expression can affect recombination frequency within crossover hotspots.

Secondary objective of this thesis was to prepare and automate bioinformatic pipelines for analysis of NGS datasets from genotyping-by-sequencing experiments, based on crossover-mapping.

Part I: Regulation of gene expression and meiotic recombination by chromatin and histone dynamics

"Efficient Generation of CRISPR/Cas9-Based Mutants Supported by Fluorescent Seed Selection in Different Arabidopsis Accessions"

Major findings of work "Efficient Generation of CRISPR/Cas9-Based Mutants Supported by Fluorescent Seed Selection in Different Arabidopsis Accessions"

CRISPR–Cas9 (clustered, regularly interspaced, palindromic region) system has been discovered for the first time as an adaptive immune system in bacteria and *Archea* (Ishino et al., 1987; Ishino et al., 2018). Adaptation in CRISPR–Cas9 system is an example of robustness and resistance toward constantly evolving bacteriophages. In this process, the viral DNA is cut, based on the presence of PAM (protospacer adjacent motif) and inserted into CRISPR locus in between the palindromic sequences as a protospacer. Cas1–Cas2–Cas4 (Cas associated) complex plays dominant role in this process (Lee et al., 2019). In the next stage, two RNA molecules are expressed. CRISPR loci are transcribed as pre-crRNA (CRISPR–related RNA), while tracRNA (trans–activating RNA) is expressed from other genomic region. The molecules form a dsRNA duplex which is then processed and cleaved. Such formed molecule consists of 76 bp of tracRNA, which serves as structural scaffold and 20 bp spacer sequence from crRNA which is necessary for homology search. In the last phase, the dsRNA duplex forms a ribonucleoprotein complex with Cas9 and activates it. In a case of subsequent bacteriophage infection, such formed RNP is guided toward viral DNA, recognizes the spacer sequence and cleaves three nucleotides upstream of PAM on both strands, causing a DSB (Barrangou & Marraffini, 2014).

After its discovery, CRISPR-Cas9 has been quickly adapted as a molecular biology tool, capable of targeted mutagenesis in plants. Previous methods used for mutation induction were based on random T-DNA insertion, chemical or physical DNA damage, virus-induced gene silencing, RNA interference and sequence-specific nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Baulcombe, 1999; Boch et al., 2009; Lloyd et al., 2005). All these methods were successfully used for the past decades, however the precision of mutagenesis, as well as high costs of large-scale screenings render them non-feasible in modern day plant research. After establishment of CRISPR/Cas9 system in human cell lines, attention was drawn to use it in plant genome editing (Jinek et al., 2012; Mali et al., 2013). Further codon optimization of Cas9 coding sequence, selection of *U3* and *U6* promoters for gRNA expression cassettes, as well as different methods for transformant screening have led to development of robust system able to induce desired, precise changes in the plant DNA sequence (Ma et al., 2016).

Since establishment of CRISPR-Cas9 in plants, multiple variations of the system emerged. With use of base editing, catalytically inactive CRISPR-Cas9 fused with deaminase can be used to modify cytosine into uracil, that during replication is read as thymine (Komor et al., 2016). Alternatively, adenine can be changed to inosine, read as guanine during DNA replication (Nishida et al., 2016). A new sophisticated method of genome editing emerged in recent years called prime editing (Anzalone et al., 2019). This approach is based on fusion of Cas9 nickase, with reverse transcriptase (Jiang et al., 2020; Lin et al., 2020). Its high specificity prompted scientists to apply this method in crops, however its poor efficiency limits the applicability of this approach for now. CRISPR-Cas9 has been also employed in epigenome editing. Fusion of catalytically inactive Cas9 (deadCas9; dCas9) with DNA methyltransferases or histone acetyltransferases was shown to efficiently modulate gene expression in plants, without permanent changes to the DNA sequence (Morita et al., 2016; Papikian et al., 2019; Paixão et al., 2019).

In this chapter, we provide basic protocol used for generation of mutants in various *Arabidopsis* accessions. Dr Tomasz Bieluszewski designed the vector and optimized method of gRNA cloning. The system described in the protocol allows for efficient generation of knock-out mutants with fast transformants screening. During the first step, a pair of 20 bp spacer sequences is designed. Then, sgRNA expression cassettes are generated by targeted mutagenesis of pJET-based vectors that contain either *AtU3* or *AtU6* promoter sequences (Addgene #173156 and #173157). The binary *pFGC-ICU2::Cas9-NSTR* vector is prepared by cloning the amplified sgRNA cassettes (Addgene #173158). Two reporter genes included in the binary vector: dsRED under *NapA* promoter and BASTA resistance gene, allow for selection of seeds under fluorescent macroscope or herbicide selection. Screening of T1 generation is based on the presence of the construct and desired mutation. However, in T2 generation, plants lacking the construct are selected, in order to segregate out the Cas9 transgene and obtain line harbouring only the desired mutation. Detailed step-by-step protocol is available in the mentioned chapter.

Within the data forming my dissertation, I utilized CRISPR/Cas9 to generate:

1) 15 distinct histone deacetylase mutants in *Arabidopsis*
2) Short-deletion mutations in the promoter of AT3G05605, gene found within the *Coco* hotspot

Based on these extensive experiments, I contributed to optimalization of the protocol, which was later included in procedure presented in the manuscript. I was also the main author of sections in the Materials and Methods chapters, related to bacteria culture, gRNA design, binary vector construction and T1 generation screening. I also participated in designing figures for this manuscript. Moreover, I was responsible for preparation of plasmid stocks sent and deposited in the AddGene, making the constructs available for scientific community.

"NuA4 and H2A.Z control environmental responses and autotrophic growth in Arabidopsis"

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Major findings of work "NuA4 and H2A.Z control environmental responses and autotrophic growth in *Arabidopsis*"

In this work we aimed to explore roles of NuA4 histone acetyltransferase complex in regulation of gene expression. To do so, we used different *epl1* mutants, which lack the AtEPL1 NuA4 subunit. EPL1, HAM1, ING2 and EAF6 comprise piccolo-NuA4 subcomplex that possesses the catalytical activity and can perform acetylation of histone and non-histone proteins in a non-directed manner. Hence, by mutating coding sequence of EPL1, we could investigate consequences of loss of NuA4 catalytical activity on gene expression.

In the *Arabidopsis* genome, there are two EPL1 orthologs, *AtEPL1A* and *ATEPL1B*. To investigate their importance, firstly a double T-DNA insertion mutant was generated (*Atepl1-1*) and later, two CRISPR-Cas9-based mutant alleles were prepared (*Atepl1-2* and *Atepl1-3* harbouring in-frame deletion). Alongside, we included *Ateaf1-2* which lacks the central NuA4 platform protein AtEAF1, however the piccolo-NuA4 maintains its non-directed acetyltransferase activity. Above-mentioned mutants display a very characteristic phenotype: they are pale-green, present retarded growth rate and chlorophyll reduction and are completely sterile (see Bieluszewski et al., 2022a Figure 1 D, Figure 2). Their phenotype reminds that of well-characterized mutants of Golden2-Like transcription factors, *glk* that have been previously found to promote chloroplast development (Fitter et al., 2002). To study possible genetic interaction between NuA4 and GLK, we used *glk* mutants in our analyses. Interestingly, the quadruple *Atepl1-2a Atepl1-2b glk1-1 glk2-1* (*Atepl1 glk*) mutant displayed aggravated phenotype effects, indicating that those factors have mostly independent functions in the transcription of genes related to growth and photosynthesis.

In order to better understand the drastic phenotype changes in above-mentioned mutants, we carried out RNA-seq experiments. For *Atepl1-2* and *Ateaf1-1*, gene expression analyses shown upregulation of 2077 and 1703 genes, respectively. On the other hand, downregulation in expression was observed 1945 and 1777 genes in *Atepl1-2* and *Ateaf1-1*, respectively. The gene ontology (GO) analysis has shown that genes related to stress response are overexpressed in NuA4 mutant backgrounds. Genes that were repressed in *Atepl1-2* and *Ateaf1-1* are associated with photosynthesis, and growth, which can explain the characteristic pale-green phenotype of the mutants.

Large proportion of both upregulated and downregulated genes were similarly dysregulated between *Atepl1-2* and *Ateaf1-1* (77.6% and 72.7%, respectively). Interestingly, *glk* transcriptome shown mostly downregulation of genes related to photosynthesis (807 uniquely mapped IDs) and overlap between *glk* and *Atepl1-2* was relatively small (31.7%). These results prove that NuA4 and GLK act independently from each other in chloroplast development (see Bieluszewski et al., 2022a Figure 3).

To further investigate mechanisms of NuA4 activity on chromatin, we decided to explore chromatin modifications over selected loci. We chose several genes, which were related to stress (*AT5G15710*), connected to chloroplast and photosynthesis (*RPS1*, *RPL11*, *RPL18* and *CHLI*) and a gene without changes in transcriptional activity in *Atepl1-2*. Additionally, we decided to include a *Gypsy* transposon, serving as a negative control. NuA4 is necessary for H4 acetylation in yeasts, so we explored levels of histone H4 acetylation (H4K5ac, H4K8ac, H4K12ac, H4K16ac) in the *Atepl1-2* background over +1 nucleosome at selected genes. As NuA4 is necessary for H2A.Z incorporation in yeasts, we examined HTA9, a major isoform of H2A.Z in *Arabidopsis* (Altaf et al., 2010). Additionally, we investigated levels of acetylated form of H2A.Z (see Bieluszewski et al., 2022a Supplementary Figure 10). All tested epigenetic modifications shown reduction in the *Atepl1-2* background, indicating the necessity of NuA4 for proper acetylation of H4 and H2A.Z. This result suggests that catalytic activity of NuA4 was also required for H2A.Z deposition.

To broaden the perspective and gain global insights into chromatin composition in *Atepl1-2*, we performed ChIP-seq experiments by profiling H4K5ac and H2A.Zac, modifications catalysed by plant NuA4. Moreover, we also investigated levels of H3K9ac that depends on other HATs, such as SAGA complex (Benhamed et al., 2006).

Additionally, we profiled total H2A.Z to understand dependence of its acetylation on chromatin deposition and histone H3 to seek for changes in nucleosome occupancy. The prepared plant chromatin has been enriched with 2% mouse chromatin (spike-in) as a calibration method for antibody specificity. Results have shown that in the *Atepl1-2* mutant, levels of total H2A.Z, H4K5ac and H2A.Zac were globally reduced. On the other hand, H3K9ac was slightly increased. Nucleosome density (H3) was higher around the TSS (see Bieluszewski et al., 2022a Figure 5 A).

Next, we aimed to link transcriptional changes in *Atepl1-2* with tested epigenetic modifications. We noticed that stress-response genes, upregulated in *Atepl1-2,* show high levels of unacetylated gene-body H2A.Z (gbH2A.Z) in wild type. Abundance of gbH2A.Z inhibits expression of these genes in non-inducible conditions. In *Atepl1-2* loss of H2A.Z from the gene-body, combined with simultaneous gain of H3K9ac at +1 nucleosome leads to their higher transcriptional activity (see Bieluszewski et al., 2022a Figure 8 A-B).

Genes related to growth and photosynthesis are downregulated in *Atepl1-2.* These genes exhibit high levels of H4K5ac and H2A.Zac at +1 nucleosome in wild type. When NuA4 is not active, global levels of these modifications are drastically reduced. This reduction is strongest for the *Atepl1-2* downregulated genes. In contrast to *Atepl1-2* overexpressed genes, no increase in H3K9ac is observed for those genes that are downregulated. These results suggest that photosynthesis, chloroplast development and growth related genes are highly dependent on NuA4-mediated H4 and H2A.Z acetylation at +1 nucleosome.

Data collected from phenotypic, transcriptomic and ChIP experiments have led us to understand the role of NuA4 in regulating gene expression in *Arabidopsis*. First, NuA4 catalyses acetylation of histone H4 and H2A.Z at +1 nucleosome of genes related to autotrophic growth, chloroplast development and photosynthesis (see Bieluszewski et al., 2022a Figure 9). This process promotes activation of expression of these crucial genes and proper plant development. Based on phenotypes of quadruple *Atepl1-2 glk* mutants and RNA-seq data, we show that NuA4 and GLK act independently from each other, but they might co-regulate the same loci. Second, NuA4 enzymatic activity is necessary for loading the H2A.Z, in its acetylated form, to gene-bodies of stress-related genes (see Bieluszewski et al., 2022a Figure 9). In wild type, histone deacetylase removes H2A.Zac mark which results in repression of responsive genes in non-

inductive conditions, caused by polymerase stalling over H2A.Z containing nucleosomes. In *Atepl1-2* however, lack of NuA4 activity precludes deposition of H2A.Z in the gene-body of stress-genes. Additionally, upregulated genes gain H3K9ac on +1 nucleosome, probably deposited opportunistically by other histone acetyltransferases. These two phenomena, lead to spontaneous activation of stress genes in *Atepl1-2*.

My role in the preparation of "NuA4 and H2A.Z control environmental responses and autotrophic growth in *Arabidopsis*" publication included conducting RNA-seq experiments (*Atepl1-2*, wild-type), performing ChIP-qPCR (H4K5ac, H4K8ac, H4K12ac, H4K16ac, H2A.Z and H2A.Zac), preparing ChIP-seq libraries (H2A.Z, H2A.Zac), inspecting the phenotype of *Atepl1-2* and to analysing and visualizing the obtained data. Additionally, I contributed to writing, designing and carrying out additional experiments, necessary for answering the reviewers questions.

HDA19 represses stress-responsive genes via H3/H2A.Z deacetylation in *Arabidopsis*
(unpublished data)

Introduction

Gene activation is particularly important during response to stress. As plants cannot escape from unfavourable environmental conditions, they evolved different pathways to counteract stress cues. One of mechanisms related to expression activation is histone acetylation catalysed by histone acetyltransferases (HATs), such as NuA4 or SAGA complexes in *Arabidopsis*. Histone acetylation leads to transcriptional activation by neutralizing the positive charge of the histone tail. Acetylated histones show lower affinity to negatively charged DNA, allowing effective binding of DNA-acting proteins such as transcription factors. For example, drought response genes were shown to be controlled by histone acetylation in wheat, poplar and *Arabidopsis* (Kim et al., 2017; Li et al., 2022; Li et al., 2019). Additionally, wound-induction genes undergo histone acetylation during callus formation (Rymen et al., 2019). Heat tolerance was also shown to be partially controlled by histone acetylation in wheat, maize and *Arabidopsis* (Lin et al., 2022; Yue et al., 2021; Zhou et al., 2024). Conversely, HAT activity is counteracted by histone deacetylases (HDACs).

In our recent report on NuA4 histone acetyltransferase complex in *Arabidopsis*, we proposed a model in which balance between HATs and HDACs leads to transcriptional repression of stress genes (Bieluszewski et al., 2022a). Catalytic activity of NuA4 is necessary for loading of H2A.Z in acetylated form in the gene-body nucleosomes of stress genes. We propose that the acetyl group is later removed, resulting in high amounts of unacetylated gbH2A.Z leading to transcriptional repression of stress genes in non-inducible conditions.

So far, no H2A.Z deacetylase has been identified in plants. To determine whether some of the HDACs are able to target H2A.Z deacetylation, we conducted a forward genetic screen of 15 histone deacetylases mutants, generated by CRISPR/Cas9. We argued that in an H2A.Z deacetylase mutant, higher levels of H2A.Zac would lead to the undesired expression of stress genes. As a result, the plant would experience reduced fitness due to the overexpression of these stress genes. Therefore, we generated *null* mutants of HDACs in *Arabidopsis* and screened the plants for changes in rosette size. We noticed that *hda19* (AT4G38130, hereafter *hda19-5*) mutant displays strongest size reduction among all the tested lines. We observed phenotypic similarities between *hda19-5* and previously well-described mutant deficient in H2A.Z loading, *arp6-1*. Moreover, we carried out 3' RNA-seq and ChIP-seq experiments to determine

transcriptomic and epigenetic changes in *hda19-5*. Based on this data, we propose a mechanism in which HDA19 specifically removes acetyl groups from H2A.Z and H3 at a subset of stress-related genes, which prevents their spontaneous expression. Targeting of HDA19 might be based on previously reported interaction with WRKY transcription factors, as promoters of upregulated *hda19-5* genes are enriched in W-box sequence (Dhatterwal et al., 2019; Kim et al., 2008).

Results

Novel CRISPR-Cas9 alleles of histone deacetylases in *Arabidopsis* display pleiotropic defects

Despite availability of T-DNA mutants for nearly all HDACs genes, we decided to generate new *null* alleles with CRISPR/Cas9. This way, we prepared a uniform collection of mutants, harbouring the deletion in the coding sequence of a selected gene, contrary to T-DNA lines with random insertion sites. With use of previously published efficient CRISPR/Cas9 protocol, we introduced out-of-frame mutations in 15 histone deacetylase genes (all Arabidopsis *HDACs* apart from *HDA8* and non-functional pseudogenes *HDA10/HDA17*) (Bieluszewski, et al., 2022b; de Rooij et al., 2021). In most cases, we induced a deletion in the first exon of HDAC genes and the generated mutations varied in size, leading to a frameshift upstream from catalytical domains. After obtaining the *null* mutants, we compared their rosette size on 28th day after germination and looked for the plants with mostly affected growth. The strongest reduction in size was observed in the *hda19-5* mutant (mean rosette size = 2.75 cm, Welch's t-test p-value = 3.82e-7; Fig. 3 A), harbouring 1 bp deletion at the cut site of Cas9, followed by a larger 47 bp deletion in the first exon, leading to a frameshift and introduction of a premature stop codon (Fig. 3 B). Other phenotype features like narrow-shaped cotyledons, early flowering and reduced fertility were in agreement with previous reports on *hda19* mutants (Ning et al., 2019; Tanaka et al., 2007). In the next step, we compared the novel *hda19-5* allele with previously well-described mutant deficient in H2A.Z loading, *arp6-1* (Choi et al., 2007; Deal et al., 2005). Both these mutants presented an early-flowering phenotype with reduced siliques, lower seed-set and smaller rosettes (Fig. 3 C-E).

Fig. 3 A Measurements of rosette size on 28th DAG. Statistical significance was determined by two-sided Welch's t-test. Significance levels: $p < 0.5$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ ****, non-significant n.s. **B** Sequencing result of *hda19-5*. Stop codons are depicted as black bars with asterisk. GRNA sequences are visualized as black arrows. **C, D, E** Analysis of flowering time (**C**), silique length (**D**) and seed set (**E**) of *hda19-5* and *arp6-1*. Statistical significance was determined with use of two-sided Welch's t-test with levels represented as described above.
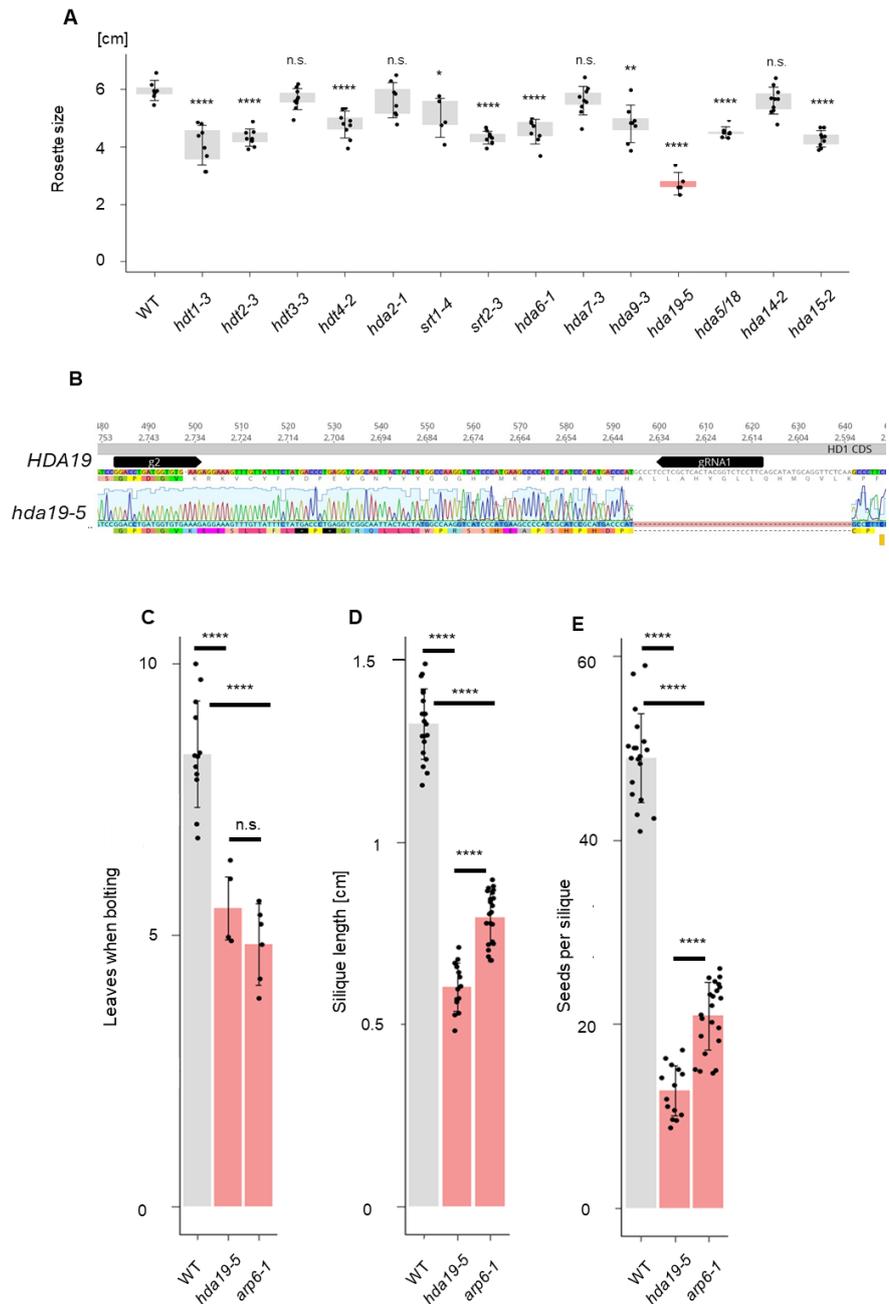
Many stress-responsive genes are upregulated in *arp6-1* as a result of global H2A.Z loss (Sura et al., 2017). We argued that H2A.Z dynamics might be also affected in the *hda19-5* background, which could lead to similar phenotype of the mutant plants. To further establish genetic interaction between *arp6-1* and *hda19-5*, we tried to obtain double homozygous mutants, however these plants were synthetically lethal ($χ^2$ test p-value = 1.36E-08; no double homozygous mutants out of 100 tested F2 plants obtained from *hda19-5 +/- arp6-1 -/-* F1 cross). These observations and previous reports on *hda19* T-DNA mutants have prompted us to investigate transcriptomic changes in the newly generated *hda19-5* (Ning et al., 2019; Shen et al., 2019).

3' RNA-seq reveals upregulation of stress genes in *hda19-5*

To investigate quantitative gene expression changes in *hda19-5*, we employed a cost-efficient 3' RNA-seq method, which is based on sequencing of 3' ends of cDNA libraries (Alpern et al., 2019; Krzyszton et al., 2024). As previous RNA-seq reports suggested a role of HDA19 in heat-stress response, we included wild type and *hda19-5* plants grown at 27°C in our analysis (Ning et al., 2019; Shen et al., 2019). In *hda19-5* grown at 22°C, 657 genes were upregulated (log2FC > 1) and 405 were downregulated (log2FC < -1) compared to wild type. Wild type plants grown at 27°C exhibited 962 upregulated and 929 downregulated genes, respectively (Fig. 4 A). To check what part of the genes misregulated in *hda19-5* are related to the response to high temperature, we compared them with genes in wild-type plants with altered expression due to heat stress (27°C). Surprisingly, as many as 437 upregulated genes in *hda19-5* were also overexpressed in wild type plants at 27°C (66%, hypergeometric test p-value < 2e-16; Fig. 4 B). The upregulated genes in both backgrounds were enriched in terms associated with response to biotic and abiotic stimuli (Fig. 4 C). Significant, though not as extensive, overlap was also observed for downregulated genes: 166 (41%) of downregulated transcripts in *hda19-5* were also underrepresented in wild type plants at 27°C (Fig. 4 B). GO terms in those groups were more distinct, usually connected with response to light and photosynthesis (Fig. 4 C). We also investigated which genes are up- and downregulated in *hda19-5* grown at 27°C, relatively to wild type at 27°C. We observed 594 upregulated genes and 341 downregulated genes in *hda19-5* at 27°C, respectively. Majority of those genes fall within previously mentioned GO terms, related to stress (Fig. 4 C). Interestingly, we found that different gene sets are dysregulated in *hda19-5* grown at 22°C compared to those grown at 27°C: Only 160 (24%) upregulated genes and 83 (24%) downregulated genes overlap between *hda19-5* at these two temperatures (Fig. 4 D). These results suggest that at control temperature (22°C), HDA19 supresses expression of large proportion of genes that normally become activated when exposed to heat stress. In *hda19-5* grown at 22°C those genes are transcriptionally induced, which might explain observed phenotype aberrancies. We speculate that in the *hda19-5* mutant background grown at 27°C, other histone deacetylases act in response to elevated temperature, activating additional stress-response genes.
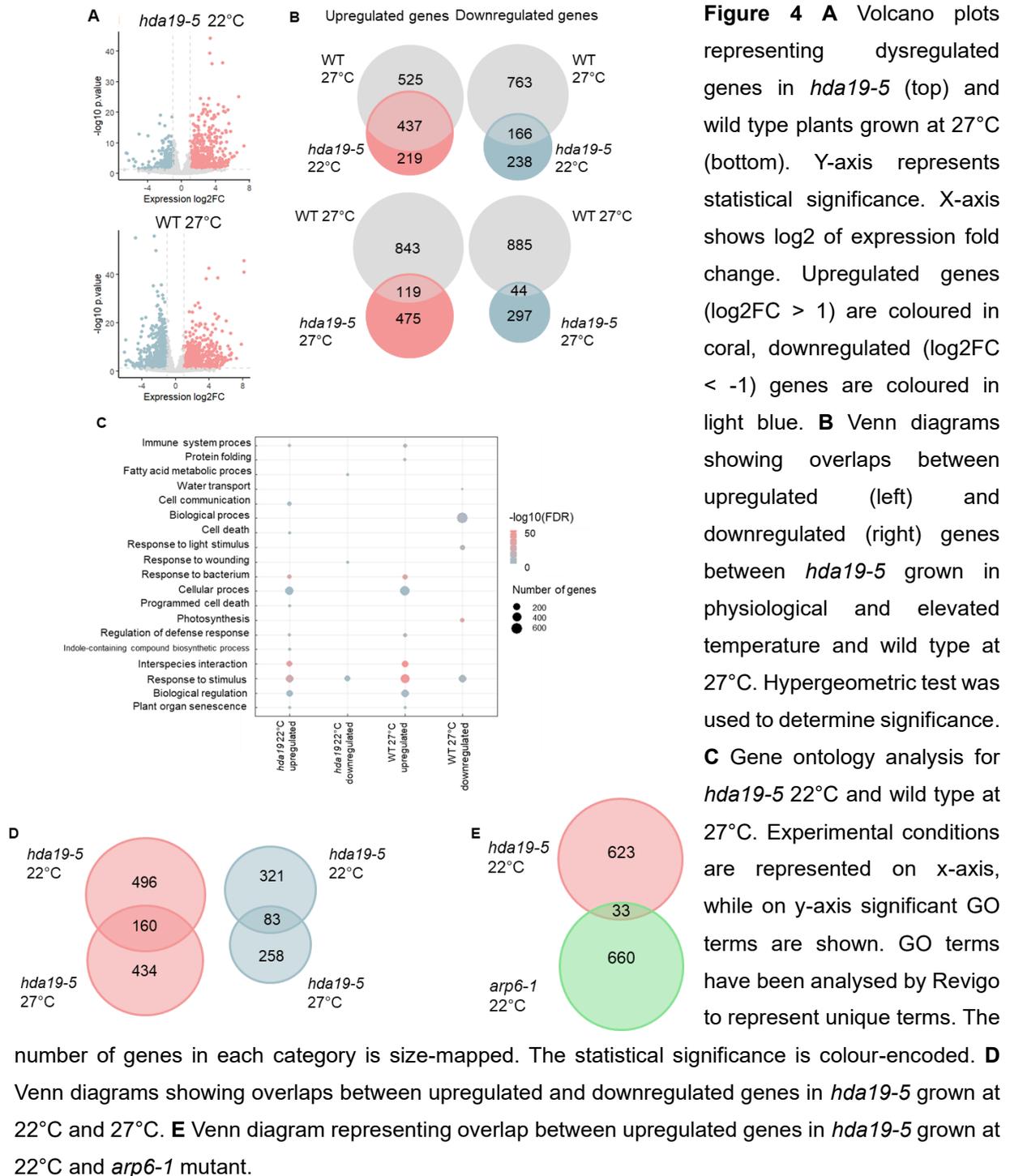
Next, we sought to determine whether transcriptomic changes in *hda19-5* at 22°C are similar to those observed in *arp6-1* mutants. We utilized a previously published RNA-seq dataset and compared the overlaps between upregulated genes (Sura et al., 2017). We found that only 33 genes are similarly upregulated in *hda19-5* (5%) and *arp6-1* (4.7%), indicating that distinct sets of genes are upregulated in these two mutants (hypergeometric test p-value = 0.02) (Fig. 4 E). This suggests that different mechanisms of chromatin-level transcriptional control are activated in *hda19-5* and *arp6-1*.

Responsive genes display hyperacetylation in *hda19-5*

Previous functional reports on HDA19 have focused on its role in H3 and H4 deacetylation (Temman & Sakamoto, 2023; Ueda et al., 2017; Zhou et al., 2013). We hypothesized that apart from its canonical histone targets, HDA19 can also catalyse H2A.Z deacetylation, causing silencing of stress-response genes. To test this, we decided to perform ChIP-seq experiments on H3K9ac, H2A.Z and H2A.Zac in the *hda19-5* background.

First, we investigated global levels of tested modifications across genes expressed in wild type plants grown at 22°C (n=19,256; genes with >10 detected transcripts in 3' RNA-seq experiment). ChIP profile indicates that a minor decrease in H3K9ac is observed at +1 nucleosome of expressed genes in *hda19-5* (p-value = 0.048). We speculate that this might be due to increased activity of other redundant histone deacetylases that compensate lack of HDA19. Interestingly, we observed a slight, though non-significant increase of H2A.Zac at +1 nucleosome in *hda19-5* (p-value = 0.10). Total levels of H2A.Z histone variant were not changed in the majority of genes in *hda19-5* background (p-value = 0.09; Fig. 5 A-C).

Lack of evident genome-wide changes in tested acetylations and H2A.Z levels in *hda19-5* prompted us to see whether particular groups of genes are differentially enriched with H3K9ac, H2A.Zac and H2A.Z (Fig. 5 D-E). To this end, we performed differential occupation analysis (log2FC > 0.6 or log2FC < -0.6) with MAnorm (Shao et al., 2012). We found that 1541 genes show hyperacetylation of H3K9ac in *hda19-5* mutant. Gene ontology analysis of these genes point out to their strong relationship with terms such as "regulation of defense response" or "response to stress" (Fig. 5 D).

Figure 4 A Volcano plots representing dysregulated genes in *hda19-5* (top) and wild type plants grown at 27°C (bottom). Y-axis represents statistical significance. X-axis shows log2 of expression fold change. Upregulated genes (log2FC > 1) are coloured in coral, downregulated (log2FC < -1) genes are coloured in light blue. **B** Venn diagrams showing overlaps between upregulated (left) and downregulated (right) genes between *hda19-5* grown in physiological and elevated temperature and wild type at 27°C. Hypergeometric test was used to determine significance. **C** Gene ontology analysis for *hda19-5* 22°C and wild type at 27°C. Experimental conditions are represented on x-axis, while on y-axis significant GO terms are shown. GO terms have been analysed by Revigo to represent unique terms. The number of genes in each category is size-mapped. The statistical significance is colour-encoded. **D** Venn diagrams showing overlaps between upregulated and downregulated genes in *hda19-5* grown at 22°C and 27°C. **E** Venn diagram representing overlap between upregulated genes in *hda19-5* grown at 22°C and *arp6-1* mutant.

On the other hand, 1795 genes displayed lower H3K9ac levels, mostly related to terms connected with response to light stimuli in the mutant background. Interestingly, statistical significance of GO enrichment for hyperacetylated genes was much stronger than genes showing lower levels of H3K9ac (Fig. 5 E). This suggests that in the

absence of HDA19, other histone deacetylases could opportunistically carry out H3 deacetylation, however without specific target preference.

Next, we found 4448 genes showing hyperacetylation of H2A.Z in *hda19-5*. These genes were associated with different categories of defence response (Fig. 5 D). Differential occupation analysis shown that 4843 genes exhibit reduced levels of H2A.Zac in the mutant background and they are mostly associated with GO terms such as "developmental process" and "tissue development" (Fig. 5 E).

Finally, we tested for differentially occupied genes by total H2A.Z in *hda19-5*. There were 4169 genes with higher occupation of H2A.Z, however the numbers of individual genes in each category, as well as statistical significance of enrichment were relatively low (Fig. 5 D). Those genes were mostly connected to terms associated with cell cycle regulation. On the other hand, only 852 genes displayed reduction of H2A.Z levels and highest number of genes belonged to "response to abiotic stimulus" GO category (Fig. 5 E).

In summary, the differential occupancy analysis led us to conclude that, despite relatively large changes in the levels of the studied modifications in both directions (hyper- and hypoacetylation), the phenotype of the *hda19* mutant – defined by the expression of stress-related genes – is primarily driven by increased acetylation of H2A.Z and H3K9.

Next, we investigated profiles of H3K9ac, H2A.Zac and H2A.Z at genes differentially enriched with these modifications in *hda19-5* background, to see which regions are mostly affected by the detected changes (Fig. 5 D-E). Metaprofiles revealed that hyperacetylated genes, show elevated H3K9ac and H2A.Zac levels over +1 nucleosome and the gene-body. Genes with higher levels of H2A.Z shown the strongest differences in the gene-body region (Fig. 6 A). Conversely, genes with detected loss of the tested modifications, show uniform decrease of H3K9ac, H2A.Zac and H2A.Z across whole gene regions (Fig. 6 B). These results suggest that genes acting in different and sometimes contrary pathways, like stress-response and development, are affected in different ways in the absence of HDA19-mediated deacetylation.

H2A.Zac is moderately increased at higher temperature in wild-type plants

We were interested in the role of H2A.Zac in gene expression regulation at higher temperature. Alongside *hda19-5*, we performed ChIP-seq experiment on wild-type plants grown at 27°C.

First, we investigated what are the profiles of H3K9ac, H2A.Z and H2A.Zac in expressed genes (the same set of genes as the one used in Fig. 5; n = 19,256). We did not notice a global increase of H3K9ac in wild type plants grown at 27°C (two-sided unpaired Wilcoxon test p-value = 0.11). We did observe however a mild increase of H2AZac at +1 nucleosome, which can be a result of elevated HAT complex activity (p = 0.003). Conversely, depletion of gbH2A.Z was observed (gene-body H2A.Z, 1250 – 2000 bp downstream from the TSS; p-value = 1.32e-11), consistent with previous reports regarding eviction of H2A.Z at high temperatures (Kumar & Wigge, 2010; van der Woude et al., 2019) (Fig. 7 A-C).

Next, we analysed genes differentially enriched with H3K9ac, H2A.Z and H2A.Zac in wild type at 27°C. The GO analysis clearly shows that individual genes showing hyperacetylation of H3K9ac (n = 1989) and H2A.Zac (n = 5325) are related to stress response, similarly to genes displaying strongest depletion of gbH2A.Z (n = 1124) in wild type at 27°C (Fig. 7 D-E). Interestingly, GO categories of discretely hyperacetylated and hypoacetylated genes in wild type at 27°C are similar to those found in *hda19-5* (Fig. 5 D-E). This, combined with similar profile of gene expression dysregulation observed in wild type at 27°C and *hda19-5*, could explain why *hda19-5* phenocopies wild-type plants grown at elevated temperature, as suggested before (Fig. 4 B) (Shen et al., 2019). We suggest that absence of HDA19 primes expression of stress-related genes via lack of H3/H2A.Z deacetylation, without the requirement of environmental stimuli.

Gene activation depends on H3K9ac, H2A.Zac and gene-body H2A.Z levels

We decided to investigate further the differences in H3K9ac, H2AZac and H2A.Z between expressed genes and those upregulated in the mutant background to better understand the cause of their dysregulation. We calculated the differences in levels of H3K9ac, H2A.Z and H2A.Zac between *hda19-5* and wild type plants, for randomly selected 2500 expressed genes and 437 genes upregulated both in *hda19-5* and in wild type plants grown at 27°C (Fig. 4 B).
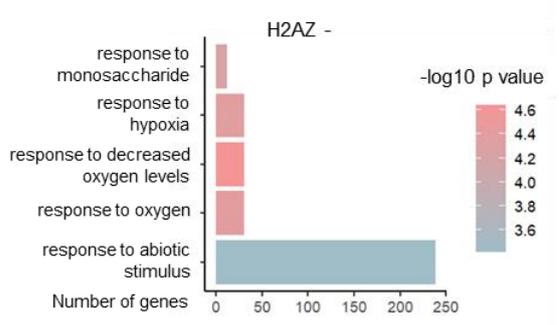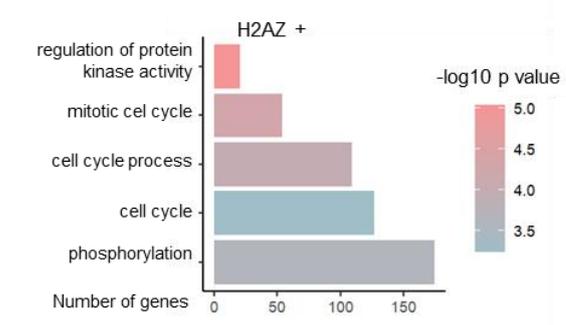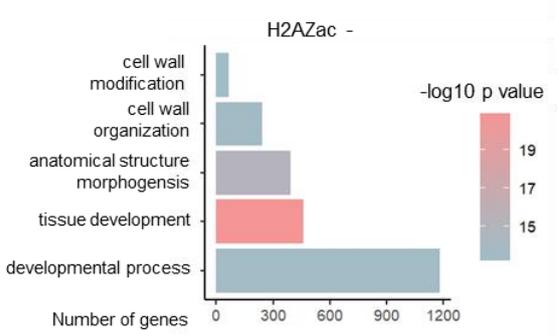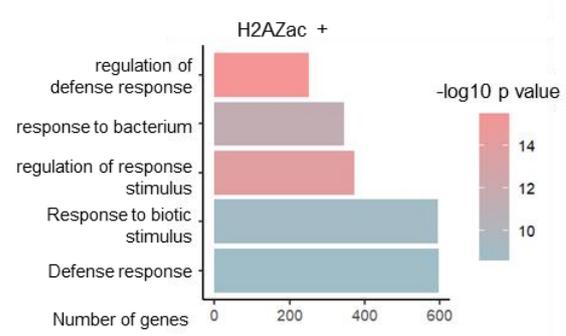
A

H3K9ac
wild type
hda19-5

B

H2AZac
wild type
hda19-5

C

H2AZ
wild type
hda19-5

D

H3K9ac +

- regulation of defense response
- regulation of response to stress
- response to fungus
- interspecies interaction between organisms
- response to stimulus

Number of genes

-log10 p value

H2AZac +

- regulation of defense response
- response to bacterium
- regulation of response stimulus
- Response to biotic stimulus
- Defense response

Number of genes

-log10 p value

H2AZ +

- regulation of protein kinase activity
- mitotic cel cycle
- cell cycle process
- cell cycle
- phosphorylation

Number of genes

-log10 p value

E

H3K9ac -

- circadian rhythm
- rhythmic process
- plastid organization
- response to light stimulus
- response to radiation

Number of genes

-log10 p value

H2AZac -

- cell wall modification
- cell wall organization
- anatomical structure morphogensis
- tissue development
- developmental process

Number of genes

-log10 p value

H2AZ -

- response to monosaccharide
- response to hypoxia
- response to decreased oxygen levels
- response to oxygen
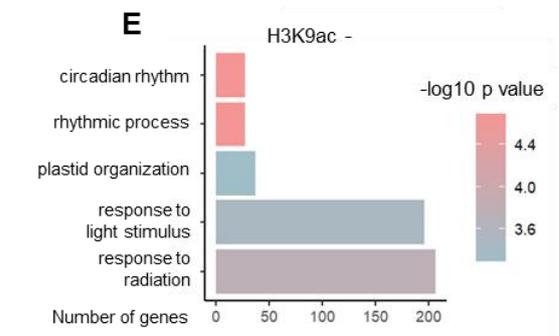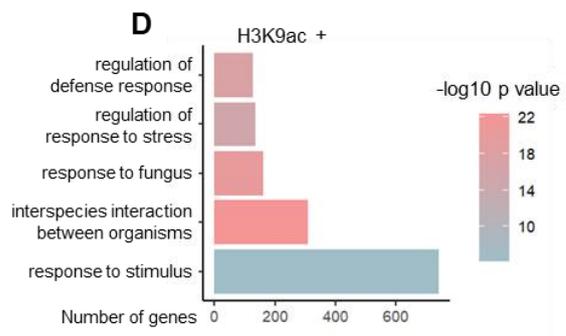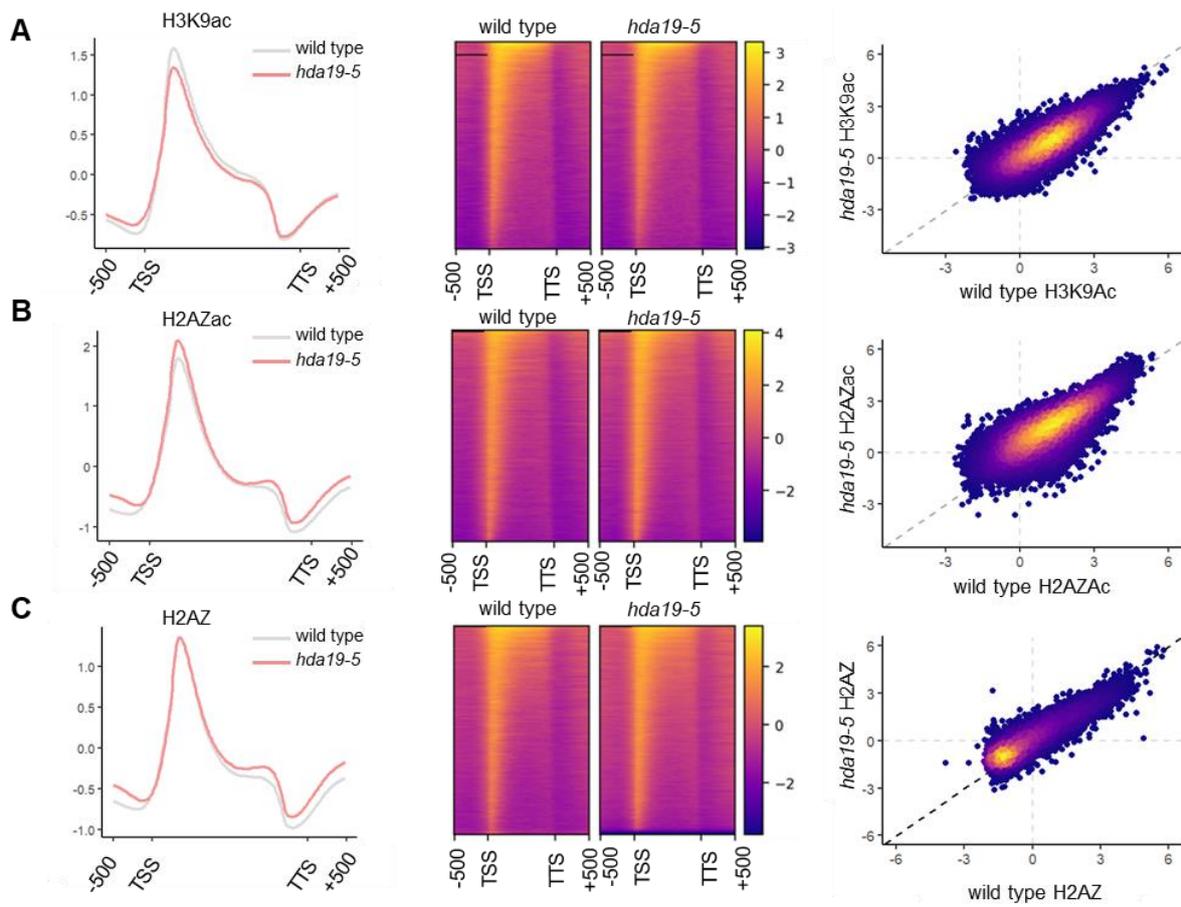- response to abiotic stimulus

Number of genes

-log10 p value

50

Figure 5 A Metagene H3K9ac signal plot for wild type (grey) and *hda19-5* (coral) overexpressed genes (n = 19,256; left). TSS marks transcription start site, TTS marks transcription termination site. Heatmaps display occupancy of H3K9ac at expressed genes in wild type and *hda19-5*. Amount of H3K9ac enrichment over input is colour-encoded (middle). Scatterplot for individual genes shows H3K9ac levels in wild type and *hda19-5*. Point density is colour-encoded (right). **B** Same as **A,** but for H2A.Zac. **C** Same as **A,** but for H2A.Z. The values on scatterplot (right) represent amounts of H2A.Z found in the gene body region. **D** Gene ontology analysis of genes showing enrichment in H3K9ac (top), H2A.Zac (middle) and H2A.Z (bottom). X-axis represents numbers of genes in each enriched category shown on y-axis. Colour of the bars reflects statistical significance (-log10 p-value of FDR). 5 most unique and abundant categories were selected for visualization. **E** Gene ontology analysis of genes showing depletion in H3K9ac (top), H2A.Zac (middle) and H2A.Z (bottom). Axes and categories were selected identically as for **D**.
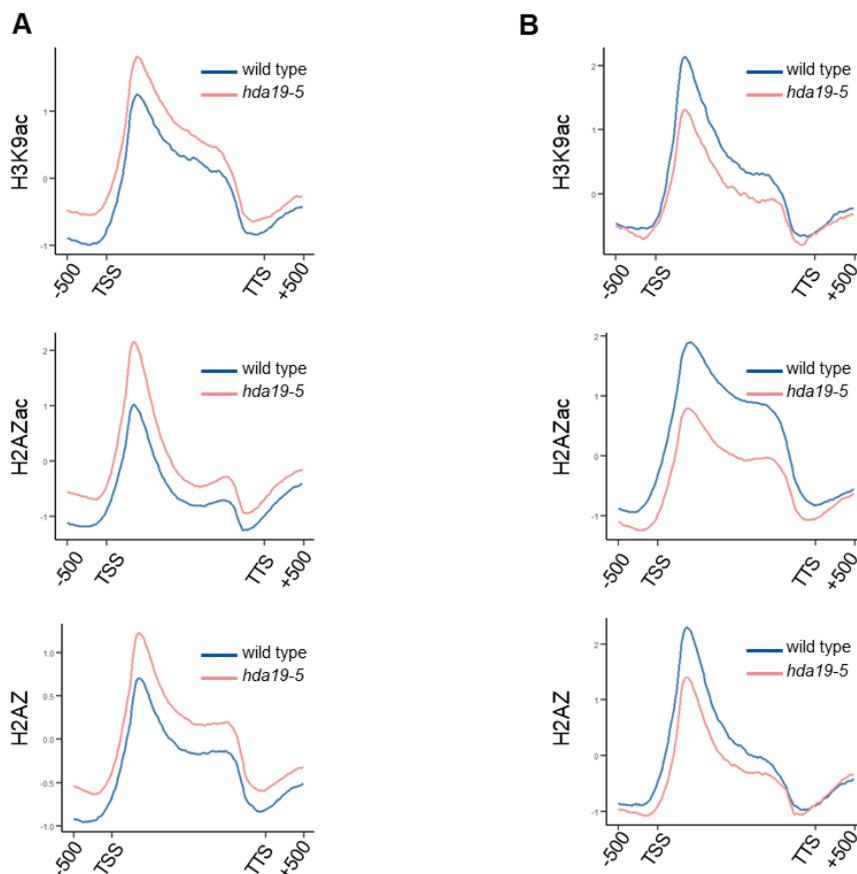

Figure 6 A Metagene plot for wild type (grey) and *hda19-5* (coral) over genes differentially enriched for H3K9ac (top), H2A.Zac (middle), H2A.Z (bottom). **B** Same as for **A,** but for genes showing depletion of tested chromatin modifications.

For acetylation marks we investigated 500 bp spanning the +1 nucleosome and for H2A.Z we analysed gene body region located 1250-2000 bp downstream from TSS. This analysis revealed that, while H3K9ac levels decrease in expressed genes in the

mutant, 437 upregulated genes show an increase in H3K9ac (two-sided unpaired Wilcoxon test, p < 2.2e-16). Furthermore, we observed a moderate increase in H2A.Zac at +1 in *hda19-5* for 437 upregulated genes (p = 0.005). In contrast, gbH2A.Z levels were not changed in upregulated genes in *hda19-5*, while only slightly elevated in control genes (p = 0.42; Fig. 8 A). These observations suggest that the overexpression found in *hda19-5* is a result of increased H3K9ac and H2A.Zac at +1 nucleosome. The levels of gbH2A.Z at *hda19*-5 upregulated genes are higher than genome-wide in wild type and *hda19-5* genetic backgrounds (p = 0.0002 and p < 2.2e-16, respectively; Fig.5 C). Abundance of gbH2A.Z prevents their spurious activation in the absence of environmental stimuli. Interestingly, levels of gbH2A.Z at upregulated genes do not loose this modification in *hda19-5* upon transcriptional activation, however, increase in +1 acetylation is enough to induce their expression. It is possible that gbH2A.Z is initially evicted during transcriptional activation, but later it is maintained by continuous replacement catalysed by SWR1-C, after initiation of gene expression (Krall & Deal, 2024).

To check whether a similar mechanism is employed in wild type at 27°C, we performed the same analysis for wild-type plants grown at different temperatures. We tested the same set of genes that are both upregulated in *hda19-5* and wild type at 27°C (n = 437). These genes gain more H3K9ac in higher temperature, but no more H2A.Zac than a set of control genes (p < 2.2e-16 and p = 0.16, respectively). Expressed genes and 437 upregulated genes loose H2A.Z from the gene-body at 27°C, however the depletion of gbH2A.Z from upregulated genes is slightly stronger (p = 0.0001, Fig. 8 B), which is in line with previous reports (Sura et al. 2017). It is important to note that the changes in H3K9ac levels between the mutant and wild type closely resemble those observed between the wild type grown at 22°C and at 27°C. However, significant changes in H2A.Zac and gbH2A.Z are only present in the comparison between the mutant and wild type. Based on this, we propose that the alterations in H2A.Zac are a direct consequence of HDA19 loss, while changes in H3 acetylation are secondary.

NuA4 and HDA19 co-regulate stress-related genes

Previously described mechanism assumed that HDAC enzyme removes acetyl group from H2A.Zac after its deposition in gene bodies mediated by NuA4, which results in transcriptional repression (Bieluszewski et al., 2022a). The *Atepl1-2* mutant, deficient

in NuA4 activity, shows global reduction of H4 and H2A.Z acetylation, as well as depletion of H2A.Z from gene-bodies due to inability to efficiently deposit H2A.Z into chromatin. Importantly, 2927 genes related to stress response are upregulated in *Atepl1-2*, which is a consequence of H2A.Z loss from their gene bodies (Bieluszewski et al., 2022a).

We compared upregulated genes in the *Atepl1-2* with those upregulated in *hda19-5*. We found that as many as 76% genes upregulated in *hda19-5* (501) were also upregulated in *Atepl1-2* (hypergeometric p-value test < 2e-16, Fig. 9 A). This is unexpected because NuA4 (*Atepl1-2*) and HDA19 (*hda19-5*) are known to have opposite effects on chromatin: NuA4 promotes histone acetylation, while HDA19 drives histone deacetylation. This also suggests that at least a part of *hda19-5* overexpressed genes may be the target of NuA4-dependent H2A.Z acetylation. We exploited our ChIP-seq data and investigated the wild type and *hda19-5* levels of H3K9ac, H2A.Z and H2A.Zac in the co-upregulated genes found in *Atepl1-2* and *hda19-5*. As those genes are included in the larger set of upregulated genes (n = 656), we expected similar profile of changes. Moreover, we re-analysed our previously published data for H3K9ac, H2A.Zac and H2A.Z in the *Atepl1-2* to understand the basis of upregulation in both mutant backgrounds, despite antagonistic roles of NuA4 and HDA19 (Bieluszewski et al., 2022a).

We tested 500 bp spanning the +1 nucleosome for histone acetylation marks, and gene-body regions for H2A.Z at co-upregulated genes shared between *Atepl1-2* and *hda19-5*. Similarly as before, we calculated differences in H3K9ac, H2A.Zac and H2A.Z levels between *hda19-5* and wild type for 501 co-overexpressed genes. Then, in the same manner, we calculated differences between *Atepl1-2* and wild type.

For the group of 501 co-upregulated genes in the *hda19-5* we noticed an increase in H3K9ac and H2A.Zac (two sided unpaired Wilcoxon test, p = 7.37e-07, p = 5.821e-11, respectively). No gain or depletion of H2A.Z was observed for the co-overexpressed genes when compared to slightly elevated levels at 2500 control genes in *hda19-5* (p = 1.306e-06). As expected, these changes were similar to those found over 437 genes overexpressed in the *hda19-5* and wild type plants at 27°C.
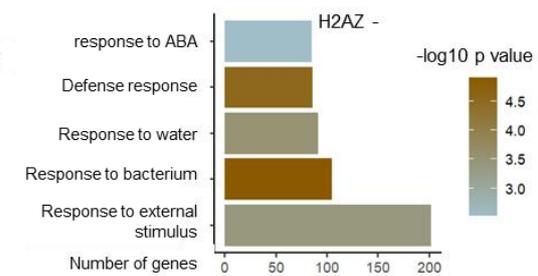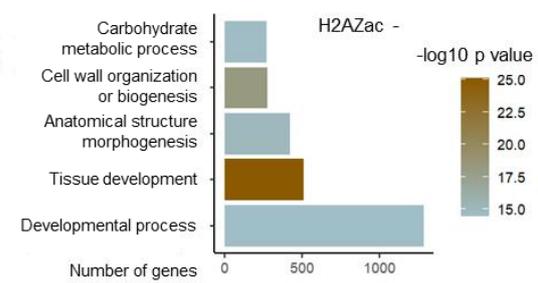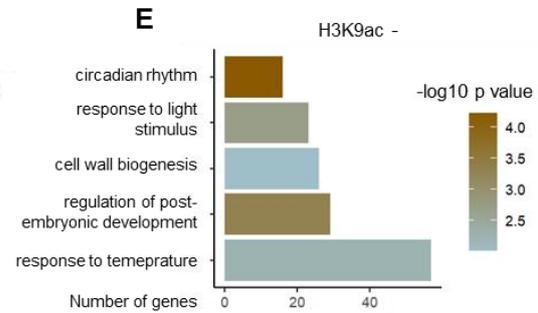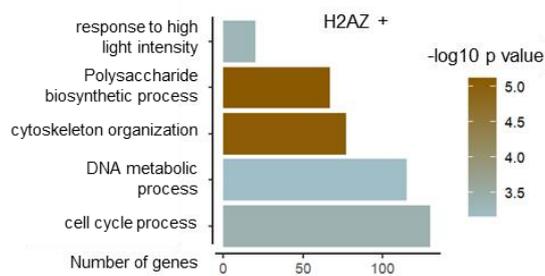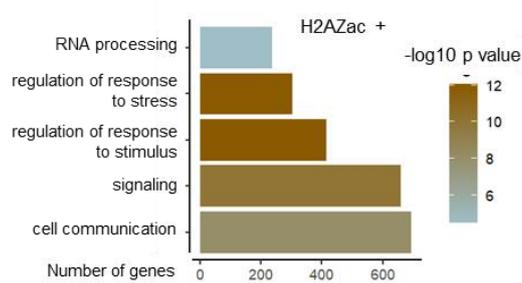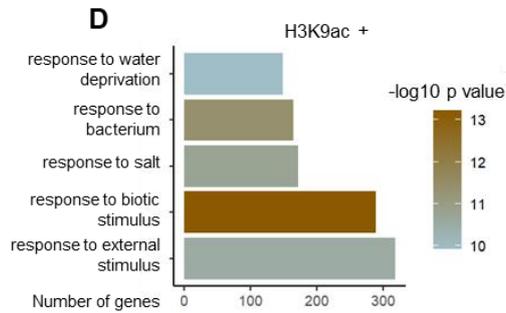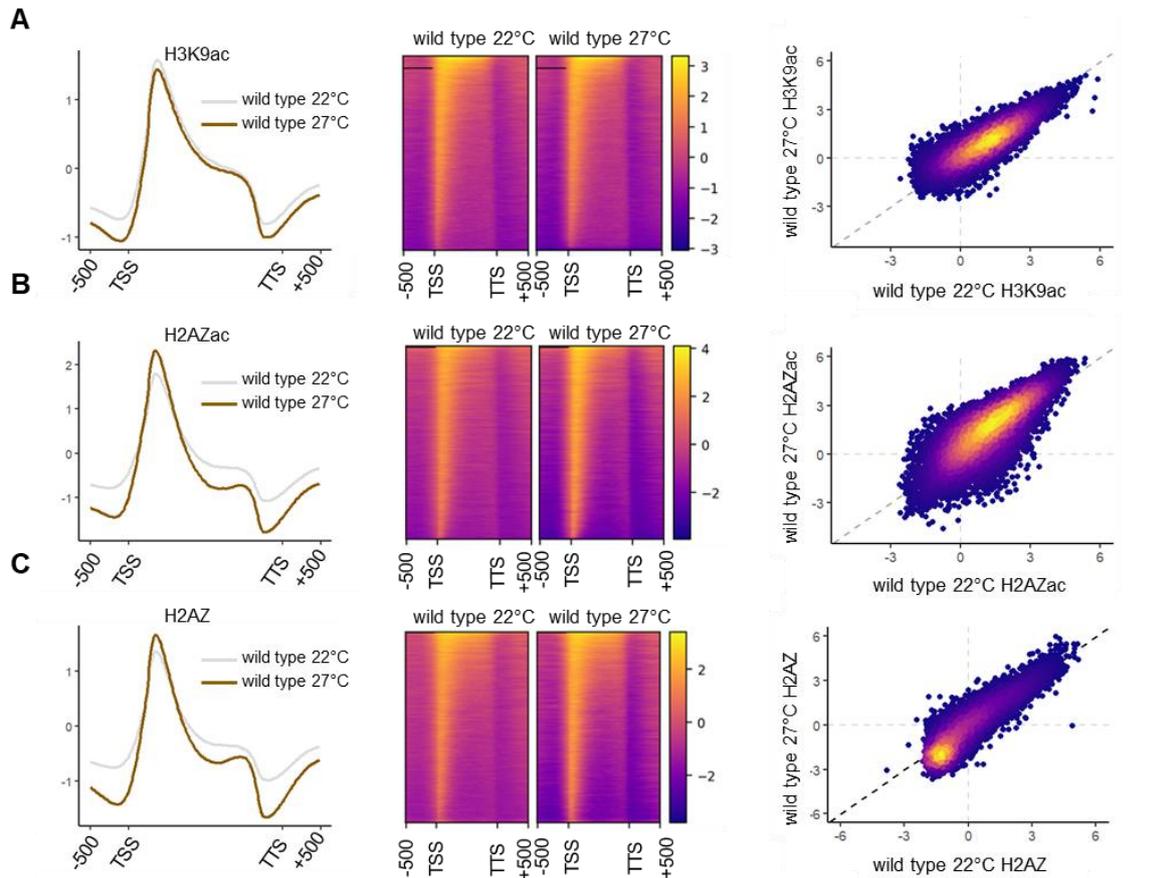
Figure 7 A Metagene H3K9ac signal plot for wild type grown at 22°C (grey) and wild type grown at 27°C (brown) overexpressed genes (n = 19,256; left). TSS marks transcription start site, TTS marks transcription termination site. Heatmaps displaying occupancy of H3K9ac at expressed genes in wild type grown at 22°C and wild type grown at 27°C. Amount of H3K9ac enrichment over input is colour encoded (middle). Scatterplot for individual genes showing H3K9ac levels in wild type grown at 22°C and wild type grown at 27°C. Point density is colour encoded (right). **B** Same as **A** but for H2A.Zac. **C** Same as **A** but for H2A.Z. The values on scatterplot (right) represent amounts of H2A.Z found in the gene body region. **D** Gene ontology analysis of genes showing enrichment in H3K9ac (top), H2A.Zac (middle) and H2A.Z (bottom). X-axis represents numbers of genes in each enriched category shown on y-axis. Colour of the bars reflects statistical significance (-log10 p-value of FDR). 5 most unique and abundant categories were selected for visualization. **E** Gene ontology analysis of genes showing depletion in H3K9ac (top), H2A.Zac (middle) and H2A.Z (bottom). Axes and categories were selected identically as for **D**.
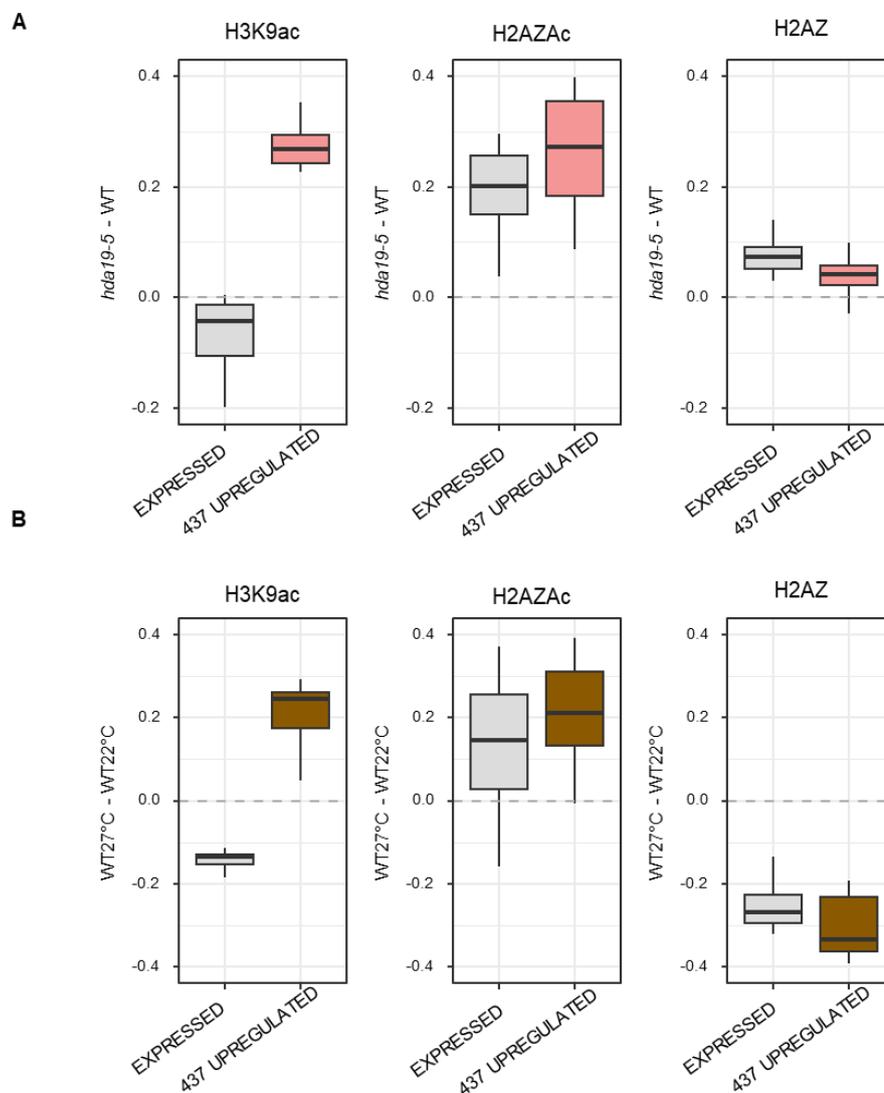
Figure 8 A Differences for H3K9ac, H2A.Z and H2A.Zac between a set of 2500 expressed genes (grey) and 437 upregulated genes in *hda19-5*. **B** Differences for H3K9ac, H2A.Z and H2A.Zac between a set of 2500 expressed genes in wild type grown at 22°C (grey) and 437 upregulated (brown) genes in wild type at 27°C.

Co-overexpressed genes shown gain of H3K9ac in the *Atepl1-2* background when compared to control genes (p = 7.801e-15). On the other hand, levels of H2A.Zac and gbH2A.Z were dramatically reduced. Interestingly, the decrease for H2A.Zac at the 501 co-upregulated genes was lower than for 2500 control genes in *Atepl1-2* (p = 2.84e-09). It is important to note that these responsive genes display low levels of H2A.Zac at +1 nucleosome in wild type and are transcriptionally inactive in non-inducible conditions (see Bieluszewski et al., 2022a Fig. 8 A). This was expected, as NuA4 is necessary for H2A.Z acetylation and deposition in the chromatin.

Those observations suggest that activation of 501 co-upregulated genes in *hda19-5* depends mostly on elevated levels of H2A.Zac and H3K9ac at +1 nucleosome, being the result of lack of HDA19-mediated histone deacetylation. In the *Atepl1-2* the same genes are overexpressed due to higher levels of H3K9ac at +1 nucleosome, probably deposited opportunistically by other histone acetylases after loss of H2A.Zac and dramatic loss of gbH2A.Z-mediated repression (Fig.9 B). This mechanism explains, why very similar group of genes is co-activated in the mutants of antagonistic enzymes.

Next, we decided to investigate genetic interactions between *hda19-5* and *Atepl1-2*. As we found evidence that HDA19 and NuA4 may stimulate expression of the same genes, we did not expect suppression of *Atepl1-2* by *hda19-5*. Indeed, we have observed that *Atepl1-2 hda19-5* showed even stronger phenotype, reducing the rosette size of *Atepl1-2* even more (Welch's t-test p = 0.015, Fig. 9 C). Additionally, triple mutant plants were extremely rare (~1/80 germinated seeds), implying that *hda19-5* has an additive effect on *Atepl1-2*, potentially leading to issues with either embryo development or germination.

Phenotype observations, together with ChIP analysis imply that a deregulation of a subset of HDA19 targets, dependent on gbH2A.Z-mediated repression, leads to additive defects in the *Atepl1-2* background.
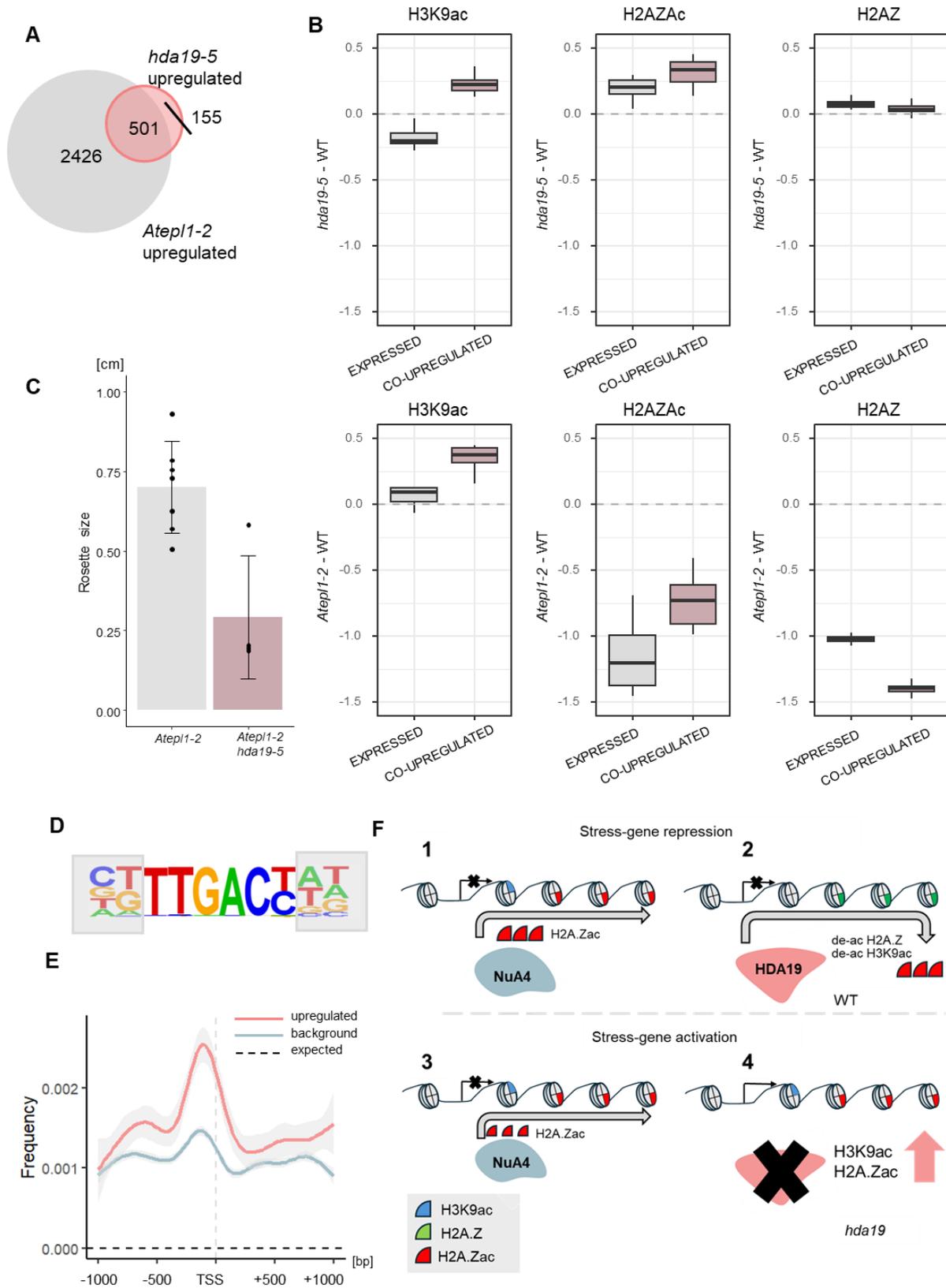
Figure 9 A Venn diagrams of overexpressed genes in *Atepl1-2* and *hda19-5.* **B** Boxplots showing differences in H3K9ac, H2A.Zac and H2A.Z for a set of control 2500 expressed genes and 501 co-

upregulated genes between *hda19-5* and wild type (top) and *Atepl1-2* and wild type (bottom) **C** Rosette sizes of *Atepl1-2*, *Atepl1-2 hda19-5*. Statistical significance was determined with two-sided Welch's t-test $p < 0.05$. **D** W-box motif found in the promoters of *hda19-5* upregulated genes. **E** Distribution of W-box motif in *hda19-5* overexpressed genes (coral) relatively to background sequences (light blue, n = 26,538) and random frequency (dotted). **F** 1) In wild type, NuA4 and SWR1 (not shown) cooperate in H2A.Zac deposition at responsive genes. 2) Subsequently, HDA19 and possibly other histone deacetylases, remove acetyl group from H2A.Z but also from core histone modifications like H3K9ac. In the end, absence of excessive histone acetylation on the +1 nucleosome and abundance of unacetylated H2A.Z in the gene-body leads to transcriptional repression. 3) In *hda19-5* mutant, NuA4 and SWR1 deposit H2A.Zac in the same way as in wild type. 4) Lack of specific H3/H2A.Z deacetylation on the +1 and deacetylation of H2A.Z in gene body leads to spurious transcriptional activation of stress genes.

Finally, we aimed to find a mechanism for HDA19 recruitment to chromatin. We analysed the promoter sequences (1000 bp upstream of the TSS) of genes upregulated in *hda19-5* to identify motifs that are relatively enriched compared to control sequences (1000 bp upstream from the TSS for 26,538 genes). We discovered recurring W-box motif (T)TGAC(C/T) (p-value < 1e-5, Benjamini-Hochberg q-value = 0.0005) found in 516 (78%) upregulated gene promoters. W-box sequence has been described as DNA motif bound by WRKY transcription factors, that act as regulators of stress-response genes in *Arabidopsis* and other plant species like rice (Chen et al., 2016; Dhatterwal et al., 2019; Hussain et al., 2018; Rushton et al., 1995; Xie et al., 2005). The frequency of W-box motif at promoters of *hda19-5* upregulated genes was higher than for the background sequences (two-sided Wilcoxon test p = 3.71e-16). It was also higher than randomly distributed motif sequence (Fig. 9 D-E). The W-box motif was mostly enriched at -1 nucleosome, upstream from the TSS. Interestingly, previous reports have shown that HDA19 can interact with WRKY transcription factors *in planta* (Kim et al., 2008). It is possible, that HDA19 is recruited to a set of stress-related genes by physical interaction with WRKY transcription factors bound to W-box sequences. After the recruitment, HDA19 can catalyse H3 and H2A.Z deacetylation, leading to transcriptional repression of stress-genes in non-inductive conditions (Fig. 9 F).

Discussion

For many decades, researchers investigated influence of histone deacetylases on stress response in plants. Reports from *Arabidopsis* but also from crop species, set new possible targets in modifying the genome in order to obtain more resilient and sustainable food production chains (Du et al., 2022; Guo et al., 2017; Sani et al., 2013; Yang et al., 2018). Although the canonical H3/H4ac HDACs targets have been exploited previously, no reports about histone H2A.Z deacetylation in plants were published.

H2A.Z histone variant has been thoroughly studied in the context of heat-response (Lorković & Berger, 2017). It has been linked with chromatin accessibility regulation and transcription activation (Sura et al., 2017; Tasset et al., 2018; Wollmann et al., 2017). Here, we show a possible functional connection between histone deacetylase 19 and H2A.Z.

Results from wild type plants growing at 27°C suggest that H3K9ac combined with H2A.Z depletion play predominant role in activation of expression (Fig. 8 B). These observations align with results suggesting that interplay between both H3/H4ac and H2A.Z/ac might be needed for transcriptional activation (Bieluszewski et al., 2022a; Colino-Sanguino et al., 2019; Draker et al., 2012).

In yeasts, mutations in histone deacetylases could rescue lack of NuA4 activity (Lin et al., 2008). Additionally, previous studies in *Arabidopsis* shown that lack of GCN5, component of SAGA complex, which is responsible for transcription initiation and elongation, as well as histone H2B and H3 acetylation, could be alleviated with mutation in HDA19 (Benhamed et al., 2006; Grasser et al., 2021). The similarities in transcriptome changes (Fig. 9 A), nevertheless caused by distinct mechanisms (Fig. 9 B), suggested that NuA4 and HDA19 act in the same pathway and no suppression should be observed. Surprisingly, the phenotype of *Atepl1-2 hda19-5* shows additive effects compared to both single mutants. This combined effect may be caused by developmental defects found in single *hda19* mutants or connected to global depletion of gbH2A.Z (Feng et al., 2021; Manrique et al., 2024; Fig.8, Fig. 9 C).

It is possible, that higher order mutants of other histone deacetylases could compensate the lack of NuA4 in *Arabidopsis*. However, in contrast to SAGA complex, NuA4 has a role reaching beyond histone acetylation which promotes gene

expression. NuA4 facilitates deposition of H2A.Z by SWR1-C into gene-body that leads to transcriptional repression of responsive genes. Although catalytic activity of NuA4 is necessary, it is unknown whether H2A.Z is directly acetylated upon deposition or canonical histones are the main targets in this process. If the latter is true, then none of the histone deacetylases would be able to supress *Atepl1-2* phenotype, as acetyl groups would not be deposited in the absence of NuA4.

The levels of H3K9ac and H2A.Zac are enriched at genes related to stress response in *hda19-5*, which might be the effect of lack of HDA19-mediated deacetylation (Fig. 5 D). However, only a fraction of genes with elevated H3K9ac and H2AZac levels are upregulated (Fig. 8 A). This discrepancy between 3' RNA-seq and ChIP-seq data may be a result of various limiting aspects. First, full-transcript length RNA-seq approach was shown to recognize more differentially expressed genes than 3' RNA-seq method (Ma et al., 2019). Moreover, for ChIP-seq we used H2AZac antibody raised against human H2A.Zac and some level of non-specific DNA-binding is possible during chromatin immunoprecipitation. This antibody was however shown to recognize also *Arabidopsis* homolog (Bieluszewski et al., 2022a; Crevillén et al., 2019). On the other hand, H3K9ac levels can be affected as a consequence of transcriptional activation, serving as a signal important for downstream steps of transcription process and not a trigger for transcriptional activation (Gates et al., 2017). The answer to this question, however, goes beyond the experimental capabilities we currently possess.

To sum up, our results presented in this chapter demonstrate that interplay between histone acetylation and deacetylation is necessary for precise regulation of stress response in *Arabidopsis*.

Materials and Methods

Plant growth conditions

Plants were grown on pellets in long-day conditions in 22°C or 27°C, at 70 % humidity and 80–90 µmol/(m^{2}s) PAR. Seeds were stratified in 4°C for 48 hours prior to transferring the pellets to growth chambers. The *arp6-1* mutant was a gift from prof. Kyuha Choi (Choi et al., 2007).

CRISPR-Cas9 mutagenesis of histone deacetylases

GuideRNA sequences were designed with use of CRISPOR software (Haeussler et al., 2016). The generated sequences were chosen according to their specificity, efficiency and off-target number. The gRNAs were targeted to induce DSBs within the same exon sequences, usually close to 5' end of the gene. Genetic constructs were prepared as described previously with use of ClonExpress MultiS One Step Cloning Kit (Vazyme, Cat. No. C113-02) (Bieluszewski, et al., 2022b). Columbia-0 plants were transformed by *Agrobacterium*-mediated floral dip (GV3101 strain). Resulting T1 seeds were selected under epifluorescent macroscope. Transformants were PCR-screened for the presence of construct and mutation in the targeted histone deacetylase with Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, Cat. No. F160L). Propagation of T2 plants was based on the presence of mutation and Cas9 vector was segregated out by selecting non-fluorescent seeds. The deletions found in *hda* mutants were confirmed with Sanger sequencing.

Phenotypic analysis

T3 plants were grown for 28 days in conditions described above. Representative rosettes have been collected and taped into paper sheet, which was later scanned. The measurements of the rosette diameter was carried out in the ImageJ software and Microsoft Excel. Flowering time was determined on the number of leaves at the moment when the plant developed fully open first flowers. The collected siliques were immersed in 80% ethanol for 2 days in 4°C and later photographed under the macroscope. First 10 siliques have been omitted from fertility tests.

RNA isolation and 3' RNA-seq

RNA was isolated for *hda19-5* and wild type plants grown in different temperatures with the use of RNeasy Plant Mini Kit (Qiagen, Cat. No. 74904). 250 ng of RNA was used for 3' RNA-seq library preparation. First, reverse transcription reaction was carried out with barcoded and UMI-containing oligo(dT) primers and SuperScript III (Thermo Fisher Scientific, Cat. No 12574026). cDNA was pooled and next purified with AMPure beads (Beckman Coulter, Cat. No. A63881). A second strand synthesis was performed overnight with the nick translation reaction containing 1x NEBNext Second Strand Synthesis Reaction Buffer (New England Biolabs, Cat. No. B6117S), 1 U RNase H (New England Biolabs, Cat. No. M0297L), 1 U E. coli DNA ligase I (New England

Biolabs, Cat. No. M0205L), 5 U E. coli DNA polymerase (New England Biolabs, Cat. No. M0209L) and 30 μM dNTPs. The double-stranded cDNA was again purified and tagmented with adapter B-loaded Tn5 transposase. Finally, libraries were amplified with Q5® High-Fidelity 2X Master Mix (New England Biolabs, Cat. No. M0492S) for 9 cycles, and further sequenced on NovaSeq 6000 in 2 x 100 bp PE mode. 4 samples per condition were analysed (16 total).

In our sequencing strategy, read R1 contains UMI and barcode with low-quality sequences after the run. We trim read R1 to keep only UMI and barcode. Fastq files for read R2 from the pools were demultiplexed into separate fastq files for each replicate using BRBseqTools (Alpern et al., 2019) (v 1.6) Demultiplex with parameters -p UB -UMI 14 -n 1. Read R2 of each library was trimmed to remove potential contamination with poly(A) tail using BRBseqTools (v 1.6) Trim and parameters -polyA 10 -minLength 30. Then mapped using STAR (v 2.7.8a) with parameters --sjdbOverhang 99 --outSAMtype BAM SortedByCoordinate --outFilterMultimapNmax 1 (Dobin et al., 2013). Finally, the deduplicated counts for each gene were obtained using BRBseqTools (v 1.6) CreateDGEMatrix with parameters -p UB -UMI 14 -s yes. Counts were used for differential gene expression analysis using the DESeq2 R package (Love et al., 2014). The Gene Ontology analysis has been carried out with use of PantherDB after removal of redundant terms with Revigo (Mi et al., 2013; Supek et al., 2011).

Chromatin isolation and ChIP-seq

The nuclei were isolated from 3 g of cross-linked fresh tissue per sample with use of Honda Buffer (440 mM sucrose, 25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.5 % Triton X-100, 10 mM β-ME, Roche Protein Inhibitor Cocktail, 10 mM PMSF, 40 mM spermine). Nuclei Lysis Buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1.6% SDS, Roche Protein Inhibitor Cocktail, 50 nM PMSF) was used to extract chromatin, which was later sonicated for 25 cycles (Diagenode Bioruptor). 100 μL of chromatin was later pre-cleared for 1 h in 4°C with Dynabeads Protein A (Thermo Fisher Scientific, Cat. No. 10002D). Next, chromatin was incubated with 5 μg of selected antibody (α-H3K9ac Millipore, Cat. No. 07352, α-HTA9 Agrisera, Cat. No. AS10718, α-H2A.Zac Diagenode, Cat. No. C15419173) overnight at 4°C. 25 μL of Dynabeads Protein A (Thermo Fisher Scientific, Cat. No. 10002D) were added to the samples and incubated for another hour in 4°C. Beads were washed twice with low-salt (150 mM NaCl), high-salt (500 mM NaCl), LiCl (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% Nonidet P-40, 0.5%

sodium deoxycholate, 1 mM PMSF) and TE buffers. Chromatin was eluted from the beads through incubation with 0.8 M NaHCO$_3$ and 1% SDS in 65°C. Following Proteinase K treatment and phenol/chloroform extraction, DNA was precipitated and 2 ng was used to prepare DNA libraries with MicroPlex Library Preparation Kit v2 (10-13 cycles, Diagenode Cat. No. C05010012). Alongside, 10 ng of Input DNA was used to prepare control libraries (6 cycles of amplification). Sequencing has been performed in 2 x 150 PE NovaSeq 6000 mode.

ChIP-seq data analysis

Paired-end libraries have been demultiplexed and trimmed with fastp (Chen et al., 2018). Paired end libraries have been mapped to TAIR10 reference genome with bowtie2 (Langmead & Salzberg, 2012). After obtaining the sorted bam files, duplicate reads have been removed. Replicate .bam files have been merged and transformed into BigWig for heatmaps and gene metaprofile generation with bamCompare and deepTools (Ramírez et al., 2014). MACS2 software has been used to call peaks enriched in comparison to chromatin input libraries with --keep-dup all --g 120000000 parameters (Zhang et al., 2008). Then MAnorm was used to determine differentially occupied regions on input-normalized peak file by tested modifications (Shao et al., 2012). Finally, GO analysis was carried out with ChIPpeakAnno R package, to determine groups of genes affected by differences in H3K9ac, H2A.Z and H2A.Zac (Zhu et al., 2010). GO terms have been filtered with use of Revigo (Supek et al., 2011). 2-3 libraries for each tested antibody were used in the analyses. 2 or 3 libraries per antibody were used for analyses.

DNA motif analyses

The 1000 bp sequences upstream from ATG codon for 657 upregulated genes were downloaded from TAIR (www.arabidopsis.org). Similarly, list of 26,538 1000 bp upstream gene sequences obtained from Phytozome 13 served as a background for motif enrichment test (Goodstein et al., 2012). DNA motif was searched with use of HOMER tool with -S 5 -len 12,14,16 parameters (Heinz et al., 2010). Plots have been prepared in R.

Modification of meiotic recombination by dCas9-VP64 targeting

(unpublished data)

Introduction

Structure of chromosome, chromatin condensation, types of epigenetic modifications and DNA accessibility affect crossover formation (Choi et al., 2018; Simorowski et al., 2018). Enzymatically inactive Cas9 (deadCas9, dCas9) can be fused with effector proteins, such as histone methyltransferases, acetylases or transcriptional modifiers like VP64 in order to locally alter chromatin states or modify gene expression levels (Morita et al., 2016; Papikian et al., 2019; Park et al., 2017). VP64 protein is a short, tetrameric 16 amino acid sequence coming from *Herpes simplex* that can act as a potent transcriptional activator in different eukaryotes including plants (Papikian et al., 2019; Seipel et al., 1992).

The rationale for using VP64 to target crossovers stems from extensive data indicating functional links between transcription, recombination, and DNA repair. For example, recent reports show that transcription-coupled DNA breaks can be repaired via homologous recombination pathway (Marnef et al., 2017). Another report shown that R-loops generated during transcription are differentially distributed during meiosis in budding yeast (Liu et al., 2023). Additionally, in fission yeast meiotic recombination hotspots are positioned in the vicinity of transcription factors binding DNA motifs (Mukiza et al., 2019; Wahls et al., 2008). In *Arabidopsis*, CO are often found in the gene promoter sequences (Choi et al., 2013; Lloyd, 2023). Moreover, VP64 was found to recruit histone acetyltransferases to nucleosomes (Utley et al., 1998). Those observations have prompted us to prepare a dCas9-VP64 fusion construct that could induce expression of a gene, found within recombination hotspot region in *Arabidopsis*.

In one of our recent studies, we described Extremely Short Interval Lines (ESILs) that present a set of genetic intervals, flanked with dsRED and eGFP coding sequences, which are expressed in the seed epidermis. One of the described interval, "*Chili Pepper*" (*ChP*), covers a 26 kb region with unusually high meiotic recombination frequency (9.09 cM/Mb) in the pericentromeric region on chromosome 3. Recombination measurements are based on the segregation of only green and red seeds. Within the tested *ChP* interval, there are 3 hotspots named *Aro*, *Coco* and *Nala*. The strongest hotspot, *Coco*, spans 3.5 kb and covers a 300 bp long-noncoding RNA (lncRNA) gene AT3G05605 (see Szymanska-Lejman et al., 2023 Figure 1 F). The main objective of this part of the PhD thesis was to investigate the possibility of

increasing the meiotic recombination frequency at *Coco* hotspot by targeting the dCas9-VP64 fusion protein to the promoter region of AT3G05605.

Results

Due to poor annotation of AT3G05605, a lncRNA located in the center of *Coco* hotpot, in the TAIR10 genome database, we decided to carry out 5'RACE-seq experiments to determine 5'UTR region boundaries. In order to check for ecotype variations, we included Col-0 and L*er*-0 (Landsberg *erecta;* hereafter Col and L*er*) *Arabidopsis* accessions in the analysis. We determined that the 5'UTR region was 93 bp long for both tested accessions. It was also AT-rich and contained TATA-box-like sequence (Fig. 10).
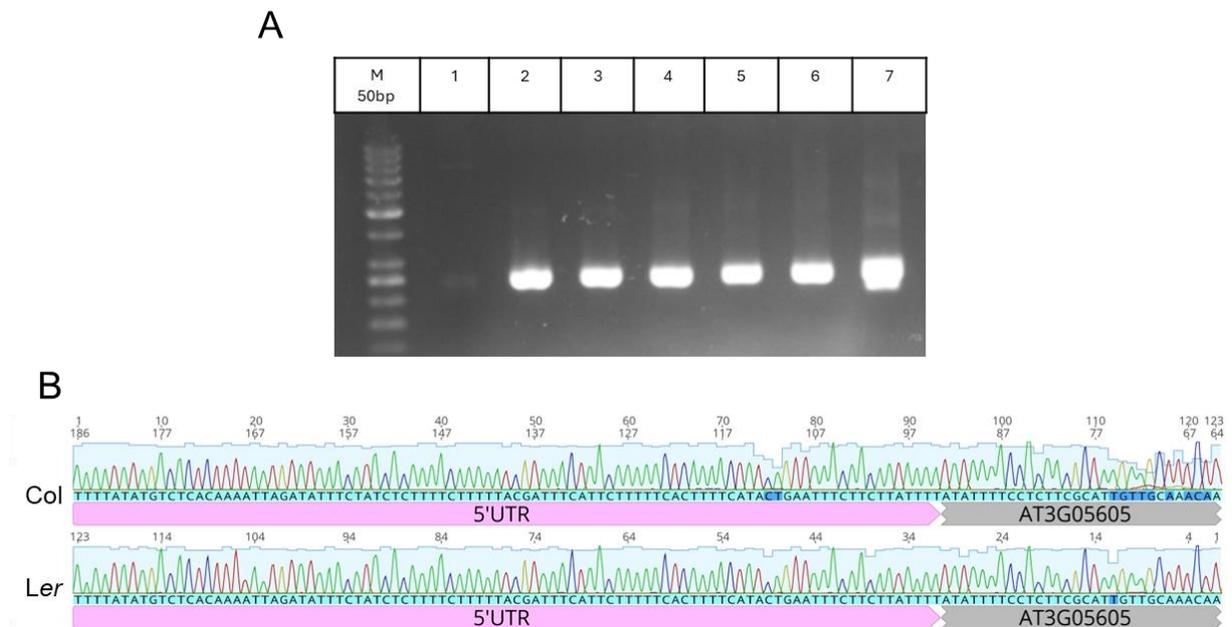
Figure 10 A. Colony PCR on bacteria harbouring plasmids with previously amplified 5' UTR region. Plasmids were isolated from colonies 2-6 and later Sanger-sequenced. **B** 5' UTR sequences of AT3G05605 for Col (upper) and L*er* (lower).

Based on the 5'UTR information, we designed single-guide RNA sequences (gRNA) that would target the dCas9-VP64 fusion protein to the promoter region of lncRNA gene. We designed 3 gRNAs, located +33 bp, +237 bp and +480 bp upstream from the TATA-like sequence. Additionally, we prepared HA-dCas9-VP64 fusion protein construct by amplifying expression cassettes and ligating them in a Gibson cloning reaction. After preparation of the genetic construct, we introduced it into Col lines

carrying *Chili Pepper* (*ChP*) via *Agrobacterium*-mediated transformation. The Col-*ChP* line that we used for this experiment was homozygous for fluorescent reporters (RG/RG). In this genetic background, measurement of recombination was impossible, since recombinant seeds would not be distinguished even if crossover occurred in *ChP* interval. Hence, after obtaining the T1 generation, transformants need to be later crossed to wild type Col or L*er* plants. This would result in plants with a hemizygous state of fluorescent reporters (RG/--), allowing the measurement of recombination frequency (RF) by calculating the ratio of red-only and green-only seeds (recombinants) to the total seed number.

The selection of transformants was based on BASTA herbicide resistance. We obtained 6 independent T1 lines and examined the expression of the targeted lncRNA gene in unopened flower buds via RT-qPCR. The dCas9-VP64 coding sequence was driven by *UBQ10* promoter (*pUBQ10)*, which ensures high levels of transgene expression in all plant tissues. In the RT-qPCR analysis, we included two reference genes, *UBC* and *ACT2*, that were used to normalize the expression levels. Line T1#3 showed nearly a 3-fold increase in AT3G05605 lncRNA expression compared to wild-type plants (Fig. 11 A). Moreover, we generated two genetic constructs: one lacking VP64 at the C-terminus of dCas9 but containing three gRNA expression cassettes, and another with the dCas9-VP64 fusion but without any gRNAs. These two vectors were used as negative controls and did not induce the expression of AT3G05605 (Fig. 11 B). We selected T1#3 individual and crossed it with Col and L*er* ecotypes as planned before. Resulting F1 generation was firstly checked with RT-qPCR to confirm that presence of genetic construct guarantees the overexpression of targeted lncRNA gene. For individuals from ChP-T1#3 x Col we obtained 4 independent F1 lines that presented stable 2.5-3.5-fold increased expression of lncRNA (coral bars on Fig. 11 C). On the other hand, F1 of ChP-T1#3 x L*er* has shown high variability, with some lines displaying overexpression and some repression of the targeted gene (light blue bars on Fig. 11 C).

The hemizygous genetic background for the eGFP and dsRED coding sequences (RG/--) in the F1 generation enabled the measurement of meiotic recombination within the *ChP* interval. We used wild-type ChP x Col and ChP x L*er* plants as controls. In the RF measurements, F1 progeny from ChP-T1#3 x Col exhibited a moderate but not statistically significant increase (Welch's t-test p-value=0.08; Fig. 11 D). Meanwhile,

hybrids from ChP-T1#3 x L*er* cross generally showed no change in meiotic recombination frequency (p-value=0.11; Fig. 11 D). At that point, given the ambiguous results from RT-qPCR and no general changes in measurements of RF for F1 ChP-T1#3 x L*er* we decided to abstain from further experiments in the hybrid background. It is possible that the presence of SNPs between Col and L*er* sequences may hinder dCas9-VP64 binding, or the recruitment of dCas9-VP64 alone was insufficient to promote the expression of the target gene. Moreover, it was not possible to conduct further experiments on the F2 progeny from ChP-T1#3 x L*er*, as these plants would exhibit a Col/L*er* mosaic genome structure due to crossovers occurring between the parents. Fortunately, those aspects were not problematic for ChP-T1#3 x Col progeny, as apart from presence of dsRED and eGFP coding sequences, the genetic background of those lines was identical. Hence, subsequent experiments were carried out on F2 plants derived from two individual F1 ChP-T1#3 x Col lines (#4 and #6).

After obtaining F2 progeny still harbouring the dCas9-VP64 construct, we investigated whether levels of increased expression of AT3G05605 are maintained. We performed RT-qPCR for the targeted lncRNA that shown 4.5-7.5 fold increase in its expression for F2#4 and F2#6, as measured in unopened flower buds (Figure 12 A). However, in case of F2#6 the overexpression was not significant due to high variation between biological replicates (Welch's t-test p-value = 0.03 and p-value = 0.12, respectively). At the same time, the *HB34* gene located in the neighbouring hotspot *Aro* (AT3G28920), shown no increase in expression in F2#4 and F2#6 (p-value = 0.16 and 0.13, respectively; Figure 12 A).

To make a functional connection between increased expression of lncRNA and presence of the construct, we carried out a ChIP-qPCR experiment to check for enrichment of dCas9-VP64 at the targeted locus. We designed two amplicons in the vicinity of gRNA binding sites in the *Coco* hotspot ("COCO2" and "COCO4") and also two control amplicons found in the neighbouring *Aro* hotspot ("ARO1" and "ARO2"). We carried out ChIP-qPCR on rosette leaves of F2#4, F2#6 and wild type plants using an HA antibody. The results clearly show that HA-dCas9-VP64 occupies the targeted recombination hotspot, but it is absent in the non-targeted region in F2 lines (two-tailed t-test p-values for F2#4 and F2#6 in "COCO2" 5.43e-05 and = 0.006, respectively; p-values for F2#4 and F2#6 in "COCO4" = 0.0039 and 0.014, respectively; Fig. 12 B).

Wild type plants showed no enrichment at either of the tested loci. This demonstrates specificity of the dCas9-VP64 targeting in the tested lines.
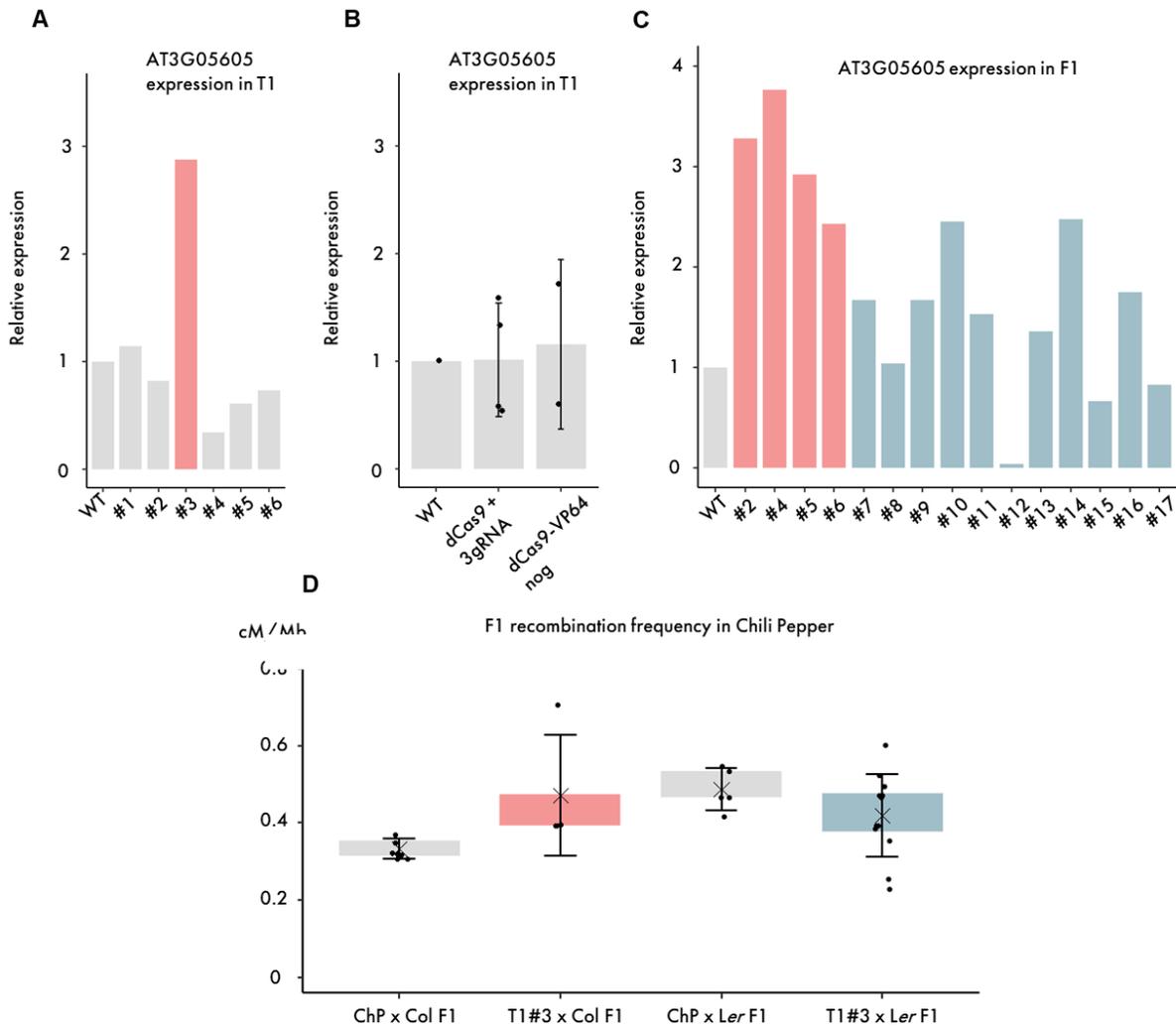

Figure 11 A Relative expression of AT3G05605 (lncRNA) gene found in *Coco* hotspot. On the x-axis, independent T1 individuals with dCas9-VP64 construct are presented. **B** Expression of AT3G05605 in T1 lines having dCas9 construct without effector protein but with gRNA cassettes and T1 lines having functional dCas9-VP64 but no gRNAs. **C** Relative expression of F1 derived from a cross between ChP-T1#3 and Col (coral) and L*er* (light blue). **D** Measurements of recombination frequency (cM) in *ChP* for F1 populations derived from a cross between ChP-T1#3 and Col and L*er*. The measurements for the plants used in **C**. Mean values for populations are marked with "X".

To investigate chromatin changes caused by targeting of dCas9-VP64 to the targeted *Coco* hotspot, we performed ChIP-qPCR on two chromatin modifications. We selected H3K4me3 and H3K27me3. First of those modifications is associated with open chromatin state and found in CO hotspots, while the latter is a hallmark for heterochromatin (Choi et al., 2018; Dai et al., 2017). To this end, we carried out ChIP-qPCR experiments and checked amplicon "COCO4" used in the HA-ChIP

experiments. In the lines with dCas9-VP64, we noticed that H3K4me3 levels were significantly increased in *COCO4* region (p-values for F2#4 and F2#6 = 0.02 and 0.01, respectively), while H3K27me3 was unaffected (p-values for F2#4 and F2#6 = 0.46 and 0.13, respectively) (Fig. 12 C).

Finally, we measured the recombination frequency in the F2 plants showing increased expression of lncRNA, enrichment of dCas9-VP64 in the targeted region and higher H3K4me3 (Fig. 12 D). The results shown significantly higher RF in the tested F2 plants (p-values for F2#4 and F2#6 = 0.008 and 0.005, respectively). These observations suggest that dCas9-VP64 can: 1) be specifically targeted to genomic locus; 2) increase expression levels of the targeted gene; 3) lead to local relaxation of chromatin that can promote CO formation.
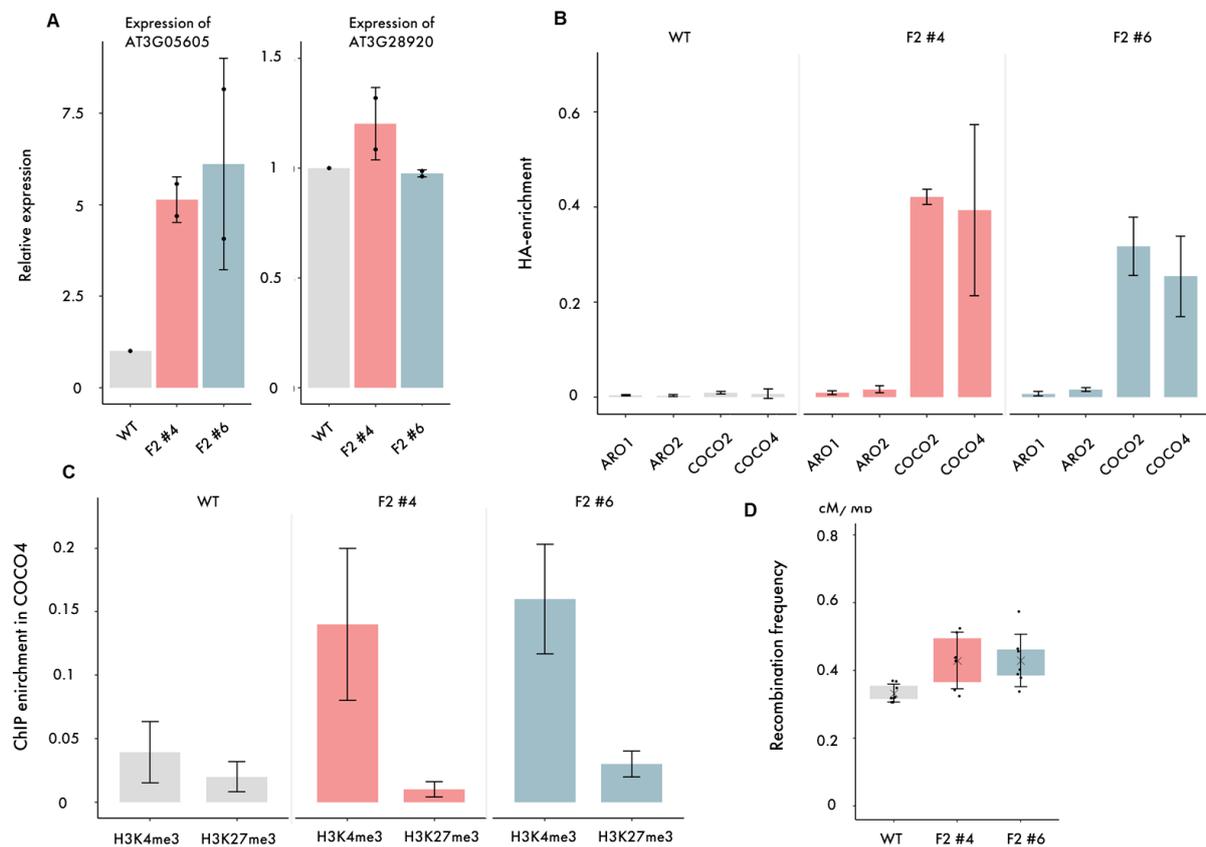
Figure 12 A Relative expression levels of AT3G05605 lncRNA (targeted gene, left) and *HB34*, AT3G28920 (non-targeted gene, right). **B** HA-dCas9-VP64 occupancy over the targeted and non-targeted locus in wild type (grey), F2#4 (coral) and F2#6 (light blue). **C** Enrichment in H3K4me3 and H3K27me3 over COCO4 amplicon (one of the presented in **B**) for wild type, F2#4 and F2#6. **D** Recombination frequency in F2#4 and F2#6 in *Chili Pepper* interval compared to ChP x Col (grey).

To further explore possibilities of modulating the recombination via alternating local intensity of gene expression, we decided to generate short deletion in close proximity of 5' UTR and TATA box of lncRNA in *Coco* hotspot. The aim of this experiment was to tamper with TFs or RNAP II binding, leading to a decrease in the expression. We designed a single gRNA located +32 bp from TATA box-like sequence and introduced Cas9 vector into ChP plants. We have found three T1 plants harbouring 26-38 bp deletions, coming from mutagenesis induced by Cas9 (Fig. 13 A). Later, we carried out RT-qPCR experiments on progeny of those lines, to see whether introduced deletion would decrease their expression levels. Unfortunately, the levels of lncRNA expression were not reduced (Fig. 13 B). One of the alternatives that could be further pursued, would be preparing the dCas9-EAR construct that could locally inhibit the transcription process, and later investigating the CO frequency (Gilbert et al., 2013).
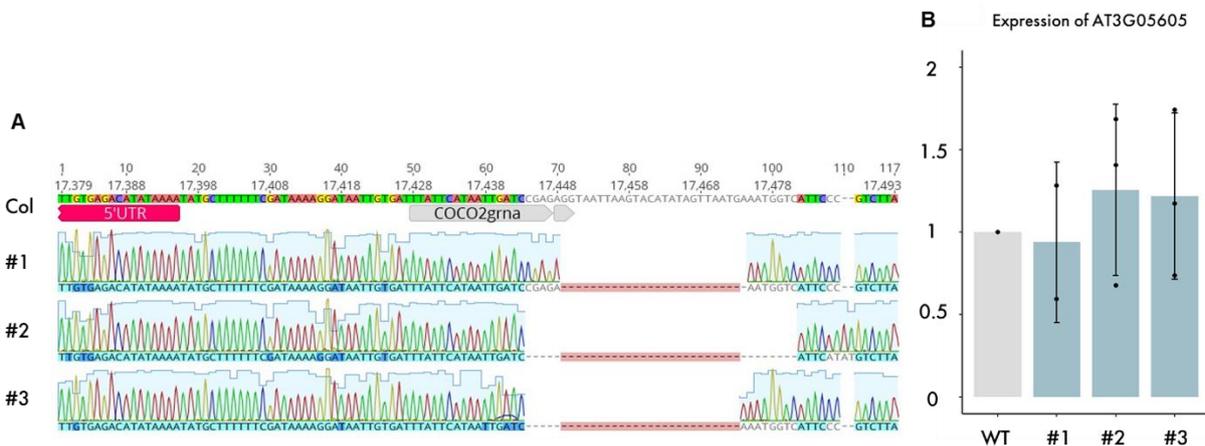
Figure 13 A Alignment of sequencing reads obtained from deletional lines. gRNA position is marked with grey bar. **B** RT-qPCR on AT3G05605 in lines with short deletions. None of the tested lines showed statistically significant change in expression of AT3G05605 gene.

Results obtained in this part of my PhD thesis show applicability of the designed dCas9-VP64 in the context of modifying the crossover formation in *Arabidopsis*. Although targeting the protein effectors, coupled with dCas9 or other DNA-binding proteins in plants has been shown previously, none of the studies demonstrated ability to stimulate crossover frequency. Results shown in here might be confirming the first use of such a genetic system.

Many further experiments, such as exchange of VP64 into another effector protein, like histone methyltransferases should be carried out in order to define robustness of the system. It should be emphasized that the *Coco* recombination hotspot is located in the

promoter of long non-coding RNA gene. In budding yeasts, the lncRNA molecules play essential roles in the formation of crossovers (Moretto et al., 2018; Rubin-Bejerano et al., 1996; Van Werven et al., 2012). As a matter of fact, distinct ncRNA species can influence the meiosis progression either by their interfering activity or in more sophisticated ways of gene and chromatin regulation in various organisms (Liu et al., 2019; Nolasco et al., 2012; Taylor et al., 2015). Whether hotspots that do not contain lncRNA can also be induced in a similar manner remains to be investigated.

Discussion

In many plant genomes, such as those of tomato or maize, the majority of recombination events occur near the ends of chromosomes, which limits the efficiency of introgressing unique traits into crop cultivars (Fuentes et al., 2022; Kianian et al., 2018). If the quantitative trait loci (QTLs) are localized in the regions with low recombination frequency, transferring them between varieties using classical breeding methods can take a lot of time and absorb many resources. A precise genetic tool, able to manipulate crossover (CO) landscape in plants would greatly accelerate crop improvement.

Previous studies in yeasts have shown that a fusion of Spo11 (a topoisomerase responsible for generation of meiotic DSBs) with Gal4 transcription factor could enhance frequency of meiotic DSB and CO formation at TF DNA-binding sites (Murakami & Nicolas, 2009; Peciña et al., 2002). Recruitment of other yeast DSB complex proteins was shown to induce local DSBs (Koehn et al., 2009). Although the targeted DSB induction works in yeast, a similar approach turned out to be unsuccessful in *Arabidopsis.* For example, an MTOPVIB topoisomerase, known to interact with SPO11-1 and SPO11-2, was fused with dCas9 in order to enhance CO frequency at *3a* CO hotspot, however this did not lead to any kind of remodelling of recombination landscape (Yelina et al., 2022). The probable reason for unsuccessful dCas9-MTOPVIB targeting, might be the fact that in *Arabidopsis*, unlike in yeasts, meiotic DSBs are formed in excess and only a fraction of them are repaired as crossovers. Induction of higher number of meiotic DSBs, a factor that does not limit CO formation, might not be the proper approach for remodelling the recombination landscape in plants.

Unsuccessful efforts of CO induction by SPO11 targeting made by other research groups, prompted us to investigate alternative methods of chromatin remodelling that in turn would promote meiotic recombination events. We chose to target VP64 peptide that was reported to attract TFs and histone acetyltransferases (Seipel et al., 1992; Utley et al., 1998). The results linking chromatin remodelling caused by dCas9-VP64 with elevated meiotic recombination rates were mostly visible in the progeny of ChP-T1#3 x Col, but not in the progeny of ChP-T1#3 x L*er*. The presence of SNPs could inhibit the efficiency of dCas9 targeting. Additionally, mosaic genetic structure of subsequent (F2) hybrid generations, could lead to variable activity of *trans*-acting CO factors like HEI10 or SNI1 (Zhu et al., 2021; Ziolkowski et al., 2017). These proteins affect recombination in different manner in Col and L*er* ecotypes genome-wide. In consequence, crossover formation in *ChP* is also affected. Moreover, in *Arabidopsis*, when the heterozygous region (HET) is adjacent to a homozygous region (HOM), more crossovers occur in the HET at the expense of reduced crossovers in the HOM (Ziolkowski et al., 2015). These changes in recombination landscape, may also affect crossover formation in *Chili Pepper*, hence majority of data presented here focus on the inbred progeny from ChP-T1#3 x Col.

Recombination frequency changes in the F2#4 and F2#6 were very moderate. To increase robustness and efficiency of the tool, the SunTag system based on recruitment of multiple copies of dCas9 coupled with effector protein could be applied (Morita et al., 2016). Additionally, VP64 could be replaced with HEI10 or other pro-crossover factors that could play more direct roles in crossover stimulation. It is also possible that use of meiosis-specific promoter, such as *pDMC1* could be more beneficial in this approach, as the chromatin remodelling would occur specifically at the meiosis stage and not beforehand in the somatic tissues.

The relationship between transcription and crossover formation is not yet clear. We observed a mild increase in expression of a lncRNA gene, located within the CO hotspot after recruitment of dCas9-VP64 to the promoter region, as well as some chromatin remodelling associated with open chromatin state. We speculate that increase in CO formation at the targeted *Chili Pepper* interval might be the result of secondary effects, connected to recruitment of various chromatin remodelling proteins that interact with VP64. The increased expression of lncRNA gene located in recombination hotspot might not have a direct effect on CO formation which aligns with

previous observations that CO are not specifically enriched in promoters of highly expressed meiotic genes in plants (Choi et al., 2018; He et al., 2017). This is however in contrary to what have been found in fission yeast, where transcription initiation sites correlate with recombination hotspots (Yamada et al., 2017). In order to explore relationship between crossover formation and gene expression, we generated mutants with short deletion upstream from 5' UTR of the lncRNA (AT3G05605) found in the *Coco* hotpot to reduce gene-expression. Unfortunately, the available PAM sites near the 5' UTR did not allow for generation of deletion that would span over TSS and completely inhibit the transcription of the lncRNA. In the future experiments, use of alternative Cas protein, which recognizes different PAM motifs may allow for better gRNA positioning and generation of deletion that influences expression of the targeted gene.

To summarize, these experiments have shown elevated recombination frequency levels in the targeted *Chili Pepper* interval, by recruitment of dCas9-VP64 to the crossover hotspot. It is yet unclear whether observed increases are an effect of direct overexpression of the targeted lncRNA gene located in the hotspot or were caused by local chromatin modification remodelling. The number of generated DSBs in the targeted interval should be also investigated, however this approach is unfeasible due to the lack of tools allowing for double strand break measurements in plants. In the future experiments, the efficiency of the system, as well as its specificity should be tested by targeting the dCas9-VP64 to another genetic interval.

Materials and Methods

5' RACE

The RNA from ~30 mg unopened flower was isolated with use of RNeasy Plant Mini Kit (Qiagen). 1 µg of RNA was used to generate cDNA with use of oligo-dT and random hexamers (HiScript III, Vazyme). We utilized 5'RACE kit (Roche) to capture the upstream regions of lncRNA by subsequent nested PCR reactions. Obtained amplicons were purified and cloned into pJET1.2 (Thermo Fisher Scientific) plasmid which was later transformed into competent DH5α *Escherichia coli* strain. Eight independent plasmid clones were sequenced for Col and L*er*.

GuideRNA design and transformants selection

GRNA sequences were manually designed based on the 5'UTR location, PAM motif presence and the nucleosome density defined by H2A.Z peaks position. 3 different sequences were used for dCas9-VP64 targeting. 1 gRNA was used for generating the small deletion in the AT3G05605 (lncRNA) promotor binding region. Transformants were selected by Basta treatment.

Molecular cloning

Expression cassettes of *pUBQ10::HA-dCas9* and *35S::BAR* were amplified with use of CloneAmp (Takara) high fidelity polymerase with addition of overhang sequences. *pCAMBIA3100* binary vector, digested with restriction enzymes was used as a backbone for Gibson cloning (ClonExpress, MultiS One Step Cloning Kit II, Vazyme).

RT-qPCR

The RNA from ~30 mg unopened flower buds were isolated with use of RNeasy Plant Mini Kit (Qiagen). 1 µg of RNA was used to generate cDNA with use of oligo-dT (HiScript III, Vazyme). Resulting cDNA was diluted 5 times and used as a template in RT-qPCR reaction (SYBR Green qPCR Master Mix, Thermo Fisher Scientific). Usually, two reference genes were used as a control (AT3G18780 and AT1G14400).

ChIP-qPCR

Chromatin was isolated from 500-700 mg of *Arabidopsis* leaves with use of Honda buffer (440 mM sucrose, 25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.5 % Triton X-100, 10 mM β-ME, Roche Protein Inhibitor Cocktail, 10 mM PMSF, 40 mM spermine). It was later pre-cleared for 1 h with Dynabeads Protein A (Thermo Fisher Scientific) and later immunoprecipitation was carried out through overnight incubation in 4 °C with 2 ng of selected antibodies (α-HA C29F4 Cell Signalling Technology, α-H3K4me3 ab8580 Abcam, α-H3K27me3 ab6002 Abcam, α-H3 ab1791 Abcam). To capture immunocomplexes, Dynabeads Protein A were added and incubated for additional 1 h in 4 °C. Then, beads were washed twice with low-salt (150 mM NaCl), high-salt (500 mM NaCl), LiCl (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF) and TE buffers. Finally, complexes were eluted by incubation with 10% Chelex. Following treatment with proteinase K, the resulting ChIP-DNA was diluted 4 times and used as a template in qPCR reactions.

Percent of input for each tested histone modifications was normalized to Input DNA and H3 levels.

Recombination frequency measurements

Seeds from hemizygous ChP (RG/--) plants were collected and cleaned with sieve filtration. Later, fluorescent macroscope (Zeiss) was used to take pictures in brightfield and with red and green fluorescence filters. The total number of seeds was counted by using in-house Python script. The number of single-color recombinants was counted manually due to low recombination rates within *ChP*. The recombination frequency was determined in cM according to the formula: $RF = 100 * (\frac{NG+NR}{NT})$ where NG equals to the number of only green seeds, NR is the number of only red seeds while NT is the total amount of seeds coming from the single plant.

"License to Regulate: Noncoding RNA Special Agents in Plant Meiosis and Reproduction"

Frontiers in Plant Sciences, 2021

Major findings of work „License to Regulate: Noncoding RNA Special Agents in Plant Meiosis and Reproduction"

The presence of long-noncoding RNA gene in the *Coco* hotspot that was selected for dCas9-VP64 targeting, prompted us to explore the roles of ncRNA species in meiosis regulation more deeply. Therefore, we prepared a review summarizing the roles of non-coding RNA in plant reproduction.

In plants, meiosis represents a convergence point for nearly all known ncRNA-dependent regulatory pathways, impacting various processes related to cell functioning and division. In the review we describe how ncRNAs have been shown to prevent transposon reactivation, establish germline-specific DNA methylation patterns and regulate the expression of meiosis-specific genes. We also discuss the influence of non-coding RNA on chromosome-level processes, such as chromosome condensation, centromere definition and crossover formation. This review article aggregates the current knowledge about ncRNAs in plant meiosis. It also brings up directions and challenges in future studies.

We start the review with description of miRNA in posttranscriptional gene silencing (PTGS) and their possible roles in controlling the expression of genes in male meiosis. Moreover, we speculate about miRNAs activity in the chromatin condensation. In the next section we focus mostly on short interfering RNA molecules (siRNA) that have been proved to act on transposon silencing and RdDM in plant meiosis (RNA-dependent DNA methylation). Subsequently we describe roles of small, uncategorized RNAs, as well as lncRNA functions in heterochromatin formation. In the summary we indicate perspectives and limitations in the ncRNA studies.

I have conceived the idea of writing this review. I wrote the first draft of the manuscript, finding the necessary literature and later participated in forming the responses to reviewers' comments. Moreover, I prepared at-a-glance summary table, containing all known ncRNA molecules acting on plant meiosis and reproduction. Additionally, I prepared all the figures presented in the manuscript.

Conclusions of Part I

1. NuA4 is a histone acetyltransferase required for H4 and H2A.Z acetylation in *Arabidopsis thaliana*. Plants lacking NuA4 display severe developmental defects.

2. Activity of NuA4 can be separated into either promoting gene expression associated with autotrophic growth or suppression of unwanted expression of stress-response genes in noninductive conditions.

3. In targeted forward genetic screen, we found HDA19 to be an important factor required for H3K9 and H2A.Z deacetylation at genes co-regulated with NuA4 acetyltransferase.

4. Plants lacking HDA19 behave as if they were grown at elevated temperatures and exhibit dysregulation of stress-related genes. This demonstrates that HDA19 is necessary to suppress stress-responsive gene expression under non-inductive conditions. The activation of responsive genes in *hda19-5* depends on H3 and H2A.Z acetylation.

5. Targeting of dCas9-VP64 is able to locally modify chromatin composition and gene expression, stimulating crossing-over formation at the targeted recombination hotspot.

Part II: Computational analyses of crossover distribution at global and fine scale in *Arabidopsis*

Introduction

Modern biological sciences vastly depend on computational techniques. Whether it is sequencing, bioimaging or mass spectrometry, bioinformatic tools are necessary for processing of large amounts of data.

"Next-generation" sequencing and recent emergence of "third-generation" platforms, allows access to genomic information applied in various analyses, such as populational or transcriptomic studies, microbial genomics or diagnostical variant calling. With the development of new technologies, design of software and implementation of algorithms, such as Hidden Markov Models (HMM) or machine learning approaches, were necessary to handle novel types of data and their unprecedented amounts. As common techniques, such as RNA-seq or ChIP-seq offer easily accessible tools and many tutorials to perform analyses, scarce protocols for genotyping-by-sequencing (GBS) were initially developed only for human genomics (Browning & Browning, 2008; Howie et al., 2009). Then, more general tools based on HMM allowed preparation of TIGER pipeline used for plant haplotype reconstruction, coming from *Arabidopsis thaliana* Col x L*er* hybrid population (Andolfatto et al., 2011; Rowan et al., 2015).

GBS experiments allow reconstruction of neighbouring blocks of haplotypes, based on sequencing data. CO detection relies on transitions between parental genotypes, which are determined by the number of sequencing reads associated with SNPs. Hence, experiments aiming to map crossovers genome-wide must be conducted in populations that exhibit DNA polymorphisms (e.g. Col x L*er* hybrids). In other words, polymorphisms have to be present in order to identify crossover breakpoint. Recent studies in the field of plant meiosis usually investigate large hybrid populations, leading to preparation of in-depth global meiotic recombination landscapes. In the experiments carried out in the Laboratory of Genome Biology, we applied TIGER pipeline, which required automatization and modifications that were necessary to meet our specific purposes. Moreover, it was needed to facilitate access to large datasets and provide a visualization tool for all members of the group. Determining crossover events at the hotspot scale was not feasible using TIGER, so it became necessary to develop a read-percentage-based method (Szymanska-Lejman et al., 2023). In this part of the doctoral thesis, I present the approaches and pipelines I developed for GBS and "seed-typing" crossover mapping.

"The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids"

Major findings of "The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids" publication

In this project, the relationship between sequence polymorphism and meiotic recombination at the CO hotspot scale has been explored. Although genome-wide associations between those factors have been investigated previously, little information is available regarding the local influence of SNPs on crossover distribution in plants (Blackwell et al., 2020; Lian et al., 2022).

During the course of experiments, Dr Maja Szymańska-Lejman has developed seed-typing method, that allows precise mapping of crossovers at the very high base-pair resolution. Dr Szymańska-Lejman generated Extremely Short Interval Lines (ESILs), that contained dsRED and eGFP fluorescent protein coding genes, expressed in seed epidermis in Col ecotype. If crossover happened between those markers, recombinant seed would display only expression of one of the fluorescent proteins that could be observed under epifluorescent stereomicroscope. On this basis, it is possible to manually select rare recombinants out of thousand of non-recombinant seeds (Col-ESIL x L*er* F$_2$ generation). After growing plants obtained from recombinant seeds, she carried out experimental procedures leading to generation of amplicon-based sequencing libraries (see Szymanska-Lejman et al., 2023 Figure 1 C-F). In the project we mapped crossovers in two different ESIL intervals: *Chili Pepper* and *BowTie* with size of 26.3 kb and 49 kb, respectively. Additionally, Col-*ChP* x L*er msh2* and Col-BT x L*er msh2* genetic backgrounds were used in seed-typing in those intervals, to determine role of MSH2 on local crossover landscape.

To determine influence of structural DNA variations and polymorphisms on CO formation, Dr Szymańska-Lejman generated crosses of ESILs with different *Arabidopsis* accessions. Whenever the structural differences between ecotypes were located outside of the recombination hotspot, no effect on CO frequency were observed. To see, whether a deletion, generated directly in the hotspot region would affect the CO formation, Dr Szymańska-Lejman generated L*er* Δ#24 line, harbouring Cas9-mediated deletion in the hottest region of the *Chili Pepper* region. This deletion mimics naturally occurring variation in C24 ecotype. F1 hybrids Col-ChP x L*er* Δ#24 and Col-ChP x C24 shown a decrease in recombination frequency when compared to

Col-ChP x L*er* (without structural variation within crossover hotspots). Additionally, in Col-ChP x Ler Δ#24 hybrids, the neighbouring hotspots (*Aro* and *Nala*), were not receiving more recombination events. The fine scale CO mapping revealed that recombination happens mostly in the regions without polymorphisms. Interestingly, in the tested L*er* and C24 populations, recombination hotspots were located in the same regions of the intervals, suggesting hotspot conservation. On the other hand, hotspots with high SNP density show a reduction in crossover formation in the background of *msh2* mutant, in which the mismatch-repair system is abrogated. These observations suggest that at local scale, MSH2 stimulates crossover CO formation.

My contribution to this work involved developing ESIL R scripts necessary for the bioinformatic analysis of amplicon-sequencing data obtained from seed-typing experiments. This approach differed from classical GBS experiments, as crossovers in the preselected recombinant plants occurred within the defined genetic interval, such as *ChP* or *BT*. In the first step, short amplicon sequencing reads were aligned to reference sequence with bwa-mem algorithm (Heng Li & Durbin, 2009). Later, SNPs were detected with samtools mpileup. Finally, based on the percentage of reads associated with the SNPs coming from reference (Col) or alternative (L*er*) sequence, the genotype was determined. Then, for each individual sample (a recombinant), a plot and output table file was created. The plot visualized genotypes as a series of connected points over every SNP position. In the region following the CO site, the percentage of Col and L*er* reads would be within the 50% (+/- 20%) range. Precise determination of CO site was possible by combined analysis of the output table file, assisted by visual inspection of individual plots (see Szymanska-Lejman et al., 2023, Supplementary Figure 2, Supplementary Table 8). This method allowed for automated analysis of 1172 individual recombinants. Additionally, it is routinely used in all seed-typing experiments carried out in the Laboratory of Genome Biology. Results of the analyses are included in a manuscript in press (Zhu et al., 2024) and in two other articles in preparation. In this work I was also responsible for handling and curating the raw data that has been uploaded to the NCBI Sequence Read Archive (SRA) under the Bio-Project accession PRJNA882919. The ESIL R script is available publicly as github repository: https://github.com/LabGenBiol/ESILs.

"MSH2 stimulates interfering and inhibits non-interfering crossovers in response to genetic polymorphism"

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Major findings of "MSH2 stimulates interfering and inhibits non-interfering crossovers in response to genetic polymorphism" publication

The aim of this work was to describe influence of DNA polymorphisms on crossover formation. One of the key factors, acting in mismatch repair is MSH2 protein. Previous studies shown that it preferentially stimulates COs in regions of higher sequence diversity, such as pericentromeres (Blackwell et al., 2020). In this manuscript, further investigation towards roles of MSH2 on formation of Class I and Class II COs was carried out.

Multiple experiments have been conducted in this research in majority by Dr Julia Dłużewska. Firstly, phenotypic observations have been made on *fancm zip4* (*fz*) and *msh2 fancm zip4* (*mfz*) Col x L*er* hybrid plants. Mutation in *ZIP4* leads to shutdown of Class I CO formation pathway, while knock-out of *FANCM* helicase, stimulates the non-interfering Class II crossovers. Interestingly, combining the mutations of *MSH2*, *FANCM* and *ZIP4*, partially rescues the fertility defects observed in the double *fz* mutant. These observations suggest that MSH2 limits Class II COs in hybrids due to its role in detecting polymorphisms between homologous chromosomes. This represents a contrasting mode of action compared to the Class I pathway, where MSH2 functions as a pro-crossover factor in regions with higher polymorphism levels (Blackwell et al., 2020; Szymanska-Lejman et al., 2023). In the subsequent experiments, including recombination frequency measurements in lines with a heterozygous block on otherwise homozygous chromosome, immunostaining, and sequencing-based whole-genome crossover mapping, we proposed a model for MSH2 activity that depends on the presence of polymorphisms. In inbred lines, MSH2 has no effect on CO formation. In Col x L*er* hybrids however, MSH2 stimulates CO formation in polymorphism-rich regions via Class I. In heterozygosity juxtaposition lines, where a heterozygous region is adjacent to a homozygous region, MSH2 is sequestered at the boundary of those segments and stimulates Class I crossover formation.

Preparation of the material used in GBS experiments is complex. First, the desired mutant genotypes in Col and L*er* backgrounds are crossed with each other to obtain F1 hybrid generation as DNA polymorphisms are required for CO determination. F1 plants are selfed, the resulting F2 seeds are sown, and the DNA is isolated from rosette leaves. Sequencing libraries are later prepared with addition of Illumina indices. Large

(>200) population of individuals needs to be sequenced to establish global distribution of CO events as each F2 plant will display a different genetic composition (see Dluzewska et al., 2023, Figure 2 A). Data from a single individual will not accurately reflect the true chromosomal positions of COs, especially in genotypes with low number of COs, such as *fz* or *mfz*. Therefore, large-scale experiments are necessary.

My involvement in this work was connected to computational analyses of GBS datasets for *fancm zip4*, *msh2 fancm*, *msh2 fancm zip4* and *msh2 recq4* Col x L*er* hybrids.

During this research I have analysed genome-wide CO distribution based on self-optimized R scripts and HMM-based TIGER software (Rowan et al., 2015). In this procedure, paired-end read sequences were aligned to a Col reference genome TAIR10 with bowtie2 and a list of SNPs was prepared using samtools software (Langmead & Salzberg, 2012; Heng Li, 2011). Resulting list of SNPs was then compared to a high quality reference list obtained from a large population of Col x L*er* F2 hybrids (Rowan et al., 2019). Finally, with some modifications in R script, the sequence data derived from each individual was subjected to TIGER pipeline to call CO sites. TIGER utilizes Hidden Markov Model (HMM) algorithms and sliding-window method to determine blocks of haplotypes across all *Arabidopsis* chromosomes (Rowan et al., 2015). I was responsible for pipelining the Bash, Perl, Java Script and R scripts into one workflow. This required some minor changes to the published scripts, but also setting up the correct working environment on Linux system. Use of computing cluster, based on Poznan Supercomputing and Networking Center, has greatly improved time and feasibility of the analysis. I also prepared a Slurm script that will allow users with little hands-on experience on running the analysis on raw files with minor changes. Additionally, I proposed visualization methods for the data included in the manuscript.

In total I analysed 1800 paired-end libraries, that were firstly aligned to Col genome reference sequence and later SNP tables were generated. Individual libraries with less than 100 000 reads associated with SNPs were discarded from the analysis (see Dluzewska et al., 2023, Supplementary Table 3. I have been also responsible for curation of the data and deposition of raw reads in the NCBI Sequence Read Archive (SRA) under PRJNA952840 number.

The high amount of sequencing data and the necessity to compare the CO distribution and numbers between different mutant genotypes, have prompted me to prepare an easy-to-access online tool, available for the members of Laboratory of Genome Biology. I wrote RShiny web application **A**rabidopsis **M**aps **o**f **R**ecombination (AMoR) that covers GBS datasets available online and those generated in our laboratory. The application is divided into two parts. First part serves as an introduction and summary of presented dataset. In the second section, the plots depicting CO distribution across 5 *Arabidopsis* chromosomes, total number of crossovers per genotype, normalized CO number across average chromosome arm and mean number of crossovers per chromosome are generated. Current version of AMOR supports comparison of up to 3 different genotypes that can be selected from drop-down menu on the left. All raw datasets are available to download. Generated plots can be customized by retyping the colours on the left panel. All R colour names are accepted, as well as HEX colour numbers. Additionally, all generated plots can be downloaded in either .pdf or .png extension. Since launching AMOR, crossover map and other plots were used in multiple publications prepared in the Laboratory of Genome Biology. Apart from the manuscript discussed in this section, AMOR facilitated preparation of two other works. The overview of AMOR is shown on Fig. 14 and the publicly available version under: **https://genomebiol.shinyapps.io/AMOR_PUBLIC/**
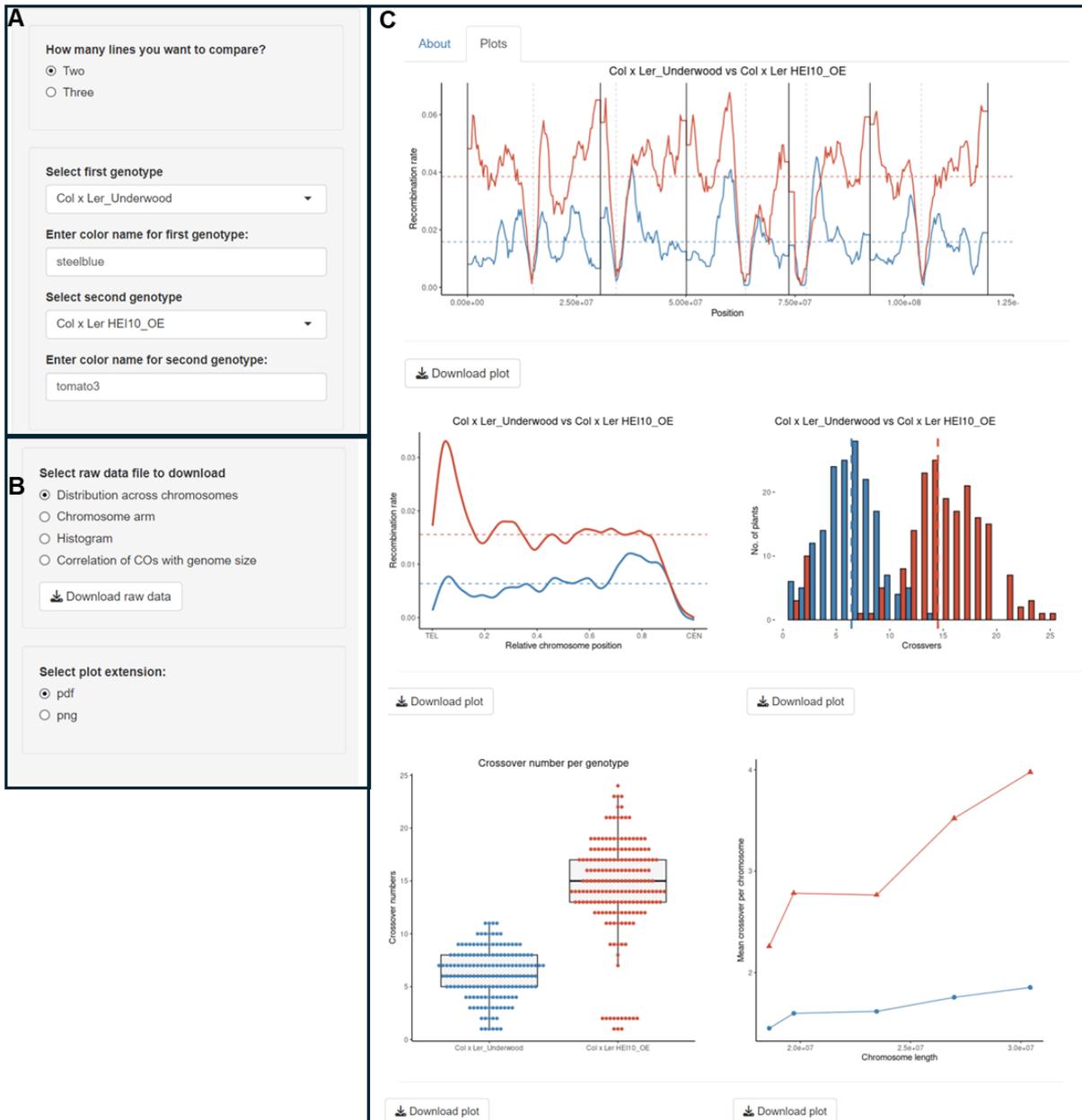
Figure 14. Overview of AMOR application. Panel "About" contains welcome message, e-mail contact address, as well as basic information about each presented dataset, such as number of F2 individuals used or source publication (not shown). Panel "Plots" is used for data visualization. Section **A** allows for selection of genotypes to present on plots and colour customization. Section **B** contains buttons for selecting the raw dataset to download and selection of plot file extension (.pdf or .png). Section **C** serves for presentation of selected datasets, such as distribution of crossovers across chromosomes (top), distribution of across averaged chromosome arm (middle left) and histogram with CO/plant numbers (middle right). Boxplots represent CO/individual (lower left) and scatter plots show CO/chromosome (lower right).

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Conclusions of Part II

1. GBS analysis serves as a fundamental method to describe the genome-wide CO distribution. This is significantly streamlined by automating crossover mapping procedures and by developing a web-based tool I created, which aggregates sequence datasets for more straightforward comparison between experiments and genotypes.

2. Seed-typing method allows for determination of crossover location with extremely high resolution. Fine scale analysis of DNA hotspots required a new pipeline, able to determine genotypes and CO sites based on reads associated with each SNP in the genetic interval. To facilitate large scale experiments I prepared automated scripts for fast seed-typing data analysis and visualization.

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Zhu, L., Dluzewska, J., Fernández-Jiménez, N., Ranjan, R., Pelé, A., Dziegielewski, W., Szymanska-Lejman, M., Hus, K., Gorna, J., Pradillo, M., & Ziolkowski, P. A. (2024). ATR controls meiotic crossover distribution at the genome scale in Arabidopsis. *The Plant Cell*.

Zhu, L., Fernández-Jiménez, N., Szymanska-Lejman, M., Pelé, A., Underwood, C. J., Serra, H., Lambing, C., Dluzewska, J., Bieluszewski, T., Pradillo, M., Henderson, I. R., & Ziolkowski, P. A. (2021). Natural variation identifies SNI1, the SMC5/6 component, as a modifier of meiotic crossover in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(33), 1–12. https://doi.org/10.1073/pnas.2021970118

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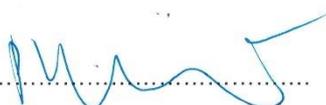
Authors contributions

Wojciech Dzięgielewski

18.07.2024, Poznań

Pracownia Biologii Genomu,

Instytut Biologii Molekularnej i Biotechnologii,

Wydział Biologii UAM

Oświadczenie współautora artykułu

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Podpis współautora

....

Podpis promotora

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*Wymienieni autorzy wnieśli równy wkład w powstanie pracy.

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Podpis promotora

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....
Podpis współautora

....
Podpis promotora

Wojciech Dzięgielewski   18.07.2024, Poznań

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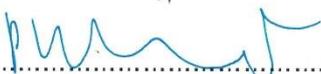
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Podpis współautora

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Podpis promotora

112

Wojciech Dzięgielewski   18.07.2024, Poznań

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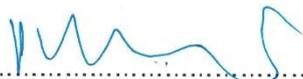
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Wydział Biologii UAM

Oświadczenie współautora artykułu

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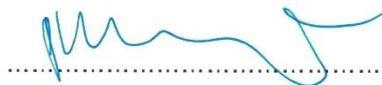..... Wojciech Dzięgielewski ....
Podpis współautora

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Prof. Piotr A. Ziółkowski   18.07.2024, Poznań

Pracownia Biologii Genomu,

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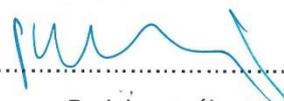..

Podpis współautora

114

Prof. Piotr A. Ziółkowski   18.07.2024, Poznań

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Instytut Biologii Molekularnej i Biotechnologii,

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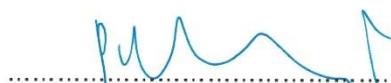*Wymienieni autorzy wnieśli równy wkład w powstanie pracy.

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Podpis współautora

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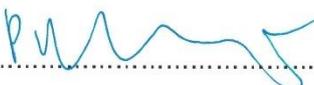
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....

Podpis współautora

Prof. Piotr A. Ziółkowski   18.07.2024, Poznań

Pracownia Biologii Genomu,

Instytut Biologii Molekularnej i Biotechnologii,

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....

Podpis współautora

Dr Julia Dłużewska   18.07.2024, Poznań

Pracownia Biologii Genomu,

Instytut Biologii Molekularnej i Biotechnologii,

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....
Podpis współautora

Dr Julia Dłużewska   18.07.2024, Poznań

Pracownia Biologii Genomu,

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Podpis współautora

120

Dr Maja Szymańska-Lejman   18.07.2024, Poznań

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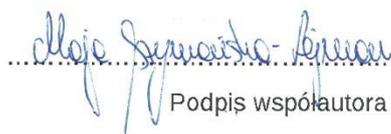
Podpis współautora

121

Dr Maja Szymańska-Lejman   18.07.2024, Poznań

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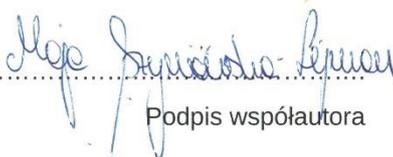*Wymienieni autorzy wnieśli równy wkład w powstanie pracy.

....
Podpis współautora

122

Dr Maja Szymańska-Lejman

18.07.2024, Poznań

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....
Podpis współautora

Dr Maja Szymańska-Lejman   18.07.2024, Poznań

Pracownia Biologii Genomu,

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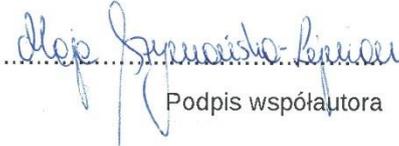
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Podpis współautora

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Oświadczenie współautora artykułu

Oświadczam, że mój udział w przygotowaniu artykułu: **Efficient Generation of CRISPR/Cas9-Based Mutants Supported by Fluorescent Seed Selection in Different Arabidopsis Accessions,** którego autorami są Tomasz Bieluszewski, Maja Szymańska-Lejman, Wojciech Dzięgielewski, Longfei Zhu oraz Piotr A. Ziółkowski opublikowanego w czasopiśmie *Methods in Molecular Biology* 2484, 161-182 (2022), doi: 10.1007/978-1-0716-2253-7_13, który jest częścią rozprawy doktorskiej Wojciecha Dzięgielewskiego polegał na: przygotowaniu wektorów i opublikowanego protokołu, przygotowaniu rysunków 1, 2 oraz 3a. Brałem udział w edycji kolejnych wersji manuskryptu.

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Oświadczenie współautora artykułu

Oświadczam, że mój udział w przygotowaniu artykułu: **NuA4 and H2A.Z control environmental responses and autotrophic growth in Arabidopsis,** którego autorami są Tomasz Bieluszewski*, Weronika Sura*, Wojciech Dzięgielewski*, Anna Bieluszewska, Catherine Lachance, Michał Kabza, Maja Szymańska-Lejman, Mateusz Abram, Piotr Włodzimierz, Nancy De Winne, Geert De Jaeger, Jan Sadowski, Jacques Côté oraz Piotr A. Ziółkowski opublikowanego w czasopiśmie *Nature Communications* 13:277 (2022), https://doi.org/10.1038/s41467-021-27882-5, który jest częścią rozprawy doktorskiej Wojciecha Dzięgielewskiego polegał na: konceptualizacji pracy, przygotowaniu linii mutantów NuA4, analizach fenotypowych, analizach RNA-seq i RT-qPCR, analizach ChIP-seq i ChIP-qPCR, badaniu i interpretacji oddziaływań białko-białko, przygotowaniu linii transgenicznych do AP-MS, analizie danych NGS oraz przygotowaniu i edycji finalnej wersji manuskryptu.

*Wymienieni autorzy wnieśli równy wkład w powstanie pracy.

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Oświadczenie współautora artykułu

Oświadczam, że mój udział w przygotowaniu artykułu: **NuA4 and H2A.Z control environmental responses and autotrophic growth in Arabidopsis,** którego autorami są Tomasz Bieluszewski*, Weronika Sura*, Wojciech Dzięgielewski*, Anna Bieluszewska, Catherine Lachance, Michał Kabza, Maja Szymańska-Lejman, Mateusz Abram, Piotr Włodzimierz, Nancy De Winne, Geert De Jaeger, Jan Sadowski, Jacques Côté oraz Piotr A. Ziółkowski opublikowanego w czasopiśmie *Nature Communications* 13:277 (2022), https://doi.org/10.1038/s41467-021-27882-5, który jest częścią rozprawy doktorskiej Wojciecha Dzięgielewskiego polegał na: przygotowaniu linii mutantów *Arabidopsis*, badaniu oddziaływań białko-białko, przygotowaniu analiz enzymatycznych oraz oczyszczaniu białek.

*Wymienieni autorzy wnieśli równy wkład w powstanie pracy.

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Oświadczam, że mój udział w przygotowaniu artykułu: **The effect of DNA polymorphisms and natural variation on crossover hotspot activity in Arabidopsis hybrids**, którego autorami są Maja Szymańska-Lejman, Wojciech Dzięgielewski, Julia Dłużewska, Nadia Kbiri, Anna Bieluszewska, R. Scott Poethig oraz Piotr A. Ziółkowski opublikowanego w czasopiśmie *Nature Communications* 14,33 (2023), https://doi.org/10.1038/s41467-022-35722-3, który jest częścią rozprawy doktorskiej Wojciecha Dzięgielewskiego polegał na przygotowaniu białka transpozazy Tn5, które zostało użyte w przygotowaniu bibliotek do seed-typing.

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18.07.2024, Poznań

Oświadczenie współautora artykułu

Oświadczam, że mój udział w przygotowaniu artykułu: **MSH2 stimulates interfering and inihibts non-interfering crossovers in response to genetic polymorphism**, którego autorami są Julia Dłużewska, Wojciech Dzięgielewski, Maja Szymańska-Lejman, Monika Gazecka, James D. Higgins, Ian R. Henderson oraz Piotr A. Ziółkowski opublikowanego w czasopiśmie *Nature Communications* 14:6716 (2023), https://doi.org/10.1038/s41467-023-42511-z, który jest częścią rozprawy doktorskiej Wojciecha Dzięgielewskiego polegał na: przygotowaniu mutantów w ekotypach Col i L*er* oraz uczestnictwie w izolacjach DNA użytego do przygotowania bibliotek do sekwencjonowania.

Monika Gazecka
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Co-author statement

I hereby declare that my contribution in preparing the article entitled: **Efficient Generation of CRISPR/Cas9-Based Mutants Supported by Fluorescent Seed Selection in Different Arabidopsis Accessions**, authored by Tomasz Bieluszewski, Maja Szymańska-Lejman, Wojciech Dzięgielewski, Longfei Zhu and Piotr A. Ziółkowski, published in *Methods in Molecular Biology* 2484, 161-182 (2022) doi: 10.1007/978-1-0716-2253-7_13, which is included in the doctoral thesis of Wojciech Dzięgielewski was cloning the *NapA::dsRED* expression cassette into pFGC binary vector.

Longfei Zhu

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Co-author signature