Investigation of methylome, transcriptome and phenotypic changes induced by temperature during asexual and sexual reproduction in the woodland strawberry (*Fragaria vesca*)

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定风波

苏轼

三月七日，沙湖道中遇雨。雨具先去，同行皆狼狈，余独不觉。已而遂晴，故作此词。

莫听穿林打叶声，何妨吟啸且徐行。竹杖芒鞋轻胜马，谁怕？一蓑烟雨任平生。

料峭春风吹酒醒，微冷，山头斜照却相迎。回首向来萧瑟处，归去，也无风雨也无晴。

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Finally, my PhD experience is the most valuable research and study experience I have ever had in my life.

Ås, September 2022.

Yupeng Zhang (张宇鹏)

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List of abbreviations

5-azaC	5- azacytidine
5mC	5-methylcytosine
A. thaliana	*Arabidopsis thaliana*
ABA	abscisic acid
AGL	AGAMOUS-LIKE
AGO	ARGONAUTE
AS	Asexual generation
AP	APETALA
ATPRMT5	Arabidopsis PROTEIN ARGININE METHYLTRANSFERASE 5
ATX	ARABIDOPSIS HOMOLOG OF TRITHORAX
ATXR7	ARABIDOPSIS TRITHORAX-RELATED7
BAH	bromo adjacent homology
BER	base excision repair
Bp	Base pair
CCA1	CIRCADIAN CLOCK ASSOCIATED1
COLDAIR	COLD ASSISTED INTRONIC NONCODING RNA
COLDWAR	COLD OF WINTER-INDUCED NONCODING RNA FROM THE PROMOTER
COOLAIR	COLD INDUCED LONG ANTISENSE INTRAGENIC RNA
CME	COLD MEMORY ELEMENT
CMT	CHROMOMETHYLASE
DCL	DICER-LIKE
DEDMG	Differentially expressed differentially methylated genes
DEDMG	Differentially expressed genes
DDM	DECREASE IN DNA METHYLATION
DHN	dehydrins
DME	DEMETER

DML	DEMETER LIKE
DMRs/DMGs	Differentially methylated regions/genes
DNA	Deoxyribonucleic Acid
DNMT	(CYTOSINE-5)-METHYLTRANSFERASE
DRM	DOMAINS REARRANGED METHYLTRANSFERASE
EBB	EARLY BUD BREAK 1
e.g.	exepli gratia; for example
EpiRILs	Epigenetic Recombinant Inbred Lines
FC	Fold-change
FT/FTL	FLOWERING LOCUS T/FLOWERING LOCUS T LIKE
FUL	FRUITFUL
F. vesca	*Fragaria vesca*
F. × ananassa	*Fragaria × ananassa*
FRI	FRIGIDA-LIKE
FUS	FUSCA
FWA	FLOWERING WAGENINGEN
GO	Gene Ontology
GRP	GLYCINE-RICH RNA-BINDING PROTEIN
H2A	Histone 2A
H3K4/H3K9/H3K27/H3K36	Histone 3 lysine 4/9/27/36
HAD	HISTONE DEACETYLASE
HEN	HUA ENHANCE
IDM	INCREASED DNA METHYLATION
Jmjc	Jumonji domain-containing
KYP	KRYPTONITE
LEC	LEAFY COTYLEDON
LFY	LEAFY
LDL	LYSINE SPECIFIC DEMETHYLASE LIKE
LHP1	LIKE HETEROCHROMATIN PROTEIN 1

LHY1	LATE ELONGATED HYPOCOTYL1
LTR	long terminal repeats
MAF4	MADS AFFECTING FLOWERING 4
MET	DNA METHYLTRANSFERASE
NERD	NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION
Paf1C	RNA POLYMERASE-ASSOCIATED FACTOR 1 COMPLEX
PEP	PERPETUAL FLOWERING
PHD	PLANT HOMEODOMAINS
Pol	RNA POLYMERASE
PRC	Polycomb Repressive Complex
PTGS	Post transcriptional gene silencing
RE	Repetitive Element
REF6	RELATIVE OF EARLY FLOWERING 6
RdDM	RNA dependent DNA methylation
RDR	RNA dependent RNA polymerase
RNA	Ribonucleic Acid
ROS	REPRESSOR OF SILENCING
SETDB1	SET Domain Bifurcated Histone Lysine Methyltransferase
siRNA	Small interfering RNA
ssRNA	single strand RNA
SHH	SAWADEE HOMEODOMAIN HOMOLOGUE
SOC	SUPPRESSOR OF CONSTANTS
SPL	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
SPT5-like	SUPPRESSOR OF TY INSERTION 5-LIKE
SRA	SET- and RING-associated
SUVH	SU(VAR)3-9 HOMOLOG
SVP	SHORT VEGETATIVE PHASE
SWN	SWINGER

TE	Transposable element
TFL	TERMINAL FLOWER
TSS/TTS	transcription start/termination site
VAL	VP1/ABI3-LIKE
VAS	VRN1 ALTERNATIVE SPLICING
VER	VERNALIZATION RELATED
VIN	VERNALIZATION INSENSITIVE 3
VRN	VERNALIZATION
VRT	VEGETATIVE TO REPRODUCTIVE TRANSITION 2

List of Papers

Paper I.

Temperature-induced methylome changes during asexual reproduction trigger transcriptomic and phenotypic changes in *Fragaria vesca*. Yupeng Zhang, Guangxun Fan, Tuomas Toivainen, Torstein Tengs, Igor Yakovlev, Paal Krokene, Timo Hytönen, Carl Gunnar Fossdal and Paul E. Grini. *Submitted manuscript*.

Paper II.

Methylome, transcriptome and phenotype change induced by temperature during sexual reproduction in *Fragaria vesca*. Yupeng Zhang, Tuomas Toivainen, Kathryn Mackenzie, Igor Yakovlev, Paal Krokene, Timo Hytönen, Paul E. Grini and Carl Gunnar Fossdal. *Manuscript*

Paper III.

Transcriptome analysis reveal major differences induced by different temperature sums during asexual and sexual reprodutions in *Fragaria vesca*. Yupeng Zhang, Marcos Viejo Somoano, Igor Yakovlev, Torstein Tengs, Paal Krokene, Timo Hytönen, Paul Grini and Carl Gunnar Fossdal. *Manuscript*

Summary

Short- and long-term environmental change is a major challenge to plants. In this thesis, I investigated the epigenetic memory of elevated temperature in different ecotypes of woodland strawberry (*Fragaria vesca*) as well as the potential machinery that establishes this memory. I determined how the *F. vesca* transcriptome and methylome adjust to elevated temperature (28 ºC vs. 18 ºC) when plants were exposed to different temperatures only during their reproduction period. This was done over three asexual generations of stolon propagation ('asexual experiment') and one sexual generation ('sexual experiment'). After three asexual generations, the elevated temperature treatment induced changes in flowering time, stolon number, and petiole length in one or more ecotypes, indicating the existence of an epigenetic memory in *F. vesca*. The ecotypes from Norway (NOR2, NOR29), Iceland (ICE2), Italy (IT4), and Spain (ES12) responded differently to temperature, with the Norwegian ecotype NOR2 showing the greatest changes in phenology and DNA methylation following asexual propagation. Only NOR2 and ES12 showed phenotypic changes following sexual propagation.

Overall DNA tended to be hypermethylated following asexual reproduction but hypomethylated following sexual reproduction. Most CHG-methylated differentially methylated regions (DMRs) were hypermethylated following asexual reproduction. Following sexual reproduction most DMRs are in the CGN context. Interestingly, only eight CHG DMR peak regions were shared across all four ecotypes used in the asexual experiment. Up to 20% of the differentially methylated genes (DMGs) showed significant gene expression changes in the asexual experiment, whereas this ratio dropped to <1% in the sexual experiment. Genes related to gibberellin metabolism, flowering time, and epigenetic modifications showed ecotype-specific methylation and expression patterns in the asexual experiment. Furthermore, repetitive elements (REs) situated within 2 Kbp of a gene correlated negatively with gene expression. Thus, in terms of DNA methylation the main finding was that elevated temperatures during asexual and sexual reproduction changed DNA methylation patterns in *F. vesca*. In addition, positional influences of REs affected gene expression, indicating that DNA methylation may be involved in general and ecotype-specific phenotypic plasticity.

We also analyzed the transcriptomic responsiveness of different ecotypes during sexual and asexual reproduction. A very small number of differentially expressed genes (DEGs) were shared between sexually reproduced and asexually reproduced individuals. In addition, most genes related to the epigenetic machinery were upregulated in the asexual progeny propagated

at 28 °C, while these genes were downregulated in the sexual progeny. This indicates that regulation of the epigenetic machinery differs between the two modes of reproduction. The Norwegian ecotype NOR2 was the most plastic ecotype at the phenotypic level, despite not having the greatest number of differentially expressed epigenetic-related genes. Taken together, my results show that an epigenetic memory to elevated temperature can be established in *F. vesca* following both asexual and sexual reproduction.

Introduction

Short- and long-term environmental change is a major challenge for plants. In times of climate change, plants must quickly adapt to a rapidly changing environment in order to maintain their fitness. The survival and competitive success of temperate plants depend on striking a good balance between utilizing the growing season to flower at the appropriate time and minimizing frost injury during the cold season. Thus, when to flower is critical and flowering time is regulated by intricate genetic networks. These networks are influenced by environmental signals that are conveyed through epigenetic mechanisms to ensure that plants flower when conditions are optimal. These epigenetic mechanisms include DNA methylation and histone modification. Epigenetic marks provide plants with phenotypic plasticity and some epigenetic marks can be directly transmitted from generation to generation via meiosis or mitosis.

Epigenetics refer to heritable expression change without any alteration of the nucleotide sequence in the genome (Turgut-Kara et al., 2020). Epigenetic regulation mechanisms include DNA methylation, non-coding RNA, histone variants, and different histone modifications such as methylation and acetylation. Epigenetic regulation can provide a mechanism for phenotypic plasticity when plants need to adjust rapidly to a changing climate, and it is hypothesized that these epigenetic changes can induce a memory and provide a solution to stress in future environments. One well-known and well-studied epigenetic memory-like mechanism in the model plant *Arabidopsis thaliana* and other flowering species, is vernalization; the memory of low temperature conditions (winter) that induces flowering. The core module of vernalization in *A. thaliana* is FLOWERING LOCUS C (FLC) (Michaels and Amasino, 1999). Numerous non-coding RNAs and histone modifications construct a complicated regulatory network of vernalization with FLC sitting at the central. However, *A. thaliana* is an annual plant with a very short life cycle, and it is therefore very different from perennial plants. The epigenetic memory of *A. thaliana* functions in a different adaptive context compared to that of perennial plants and this makes *Arabidopsis thaliana* less than an ideal model to research the epigenetics memory in perennial plants.

We chose woodland strawberry *Fragaria vesca* (2n = 2x = 14) as a model to investigate how a perennial plant responds to climate change, and specifically to learn if a temperature-induced epigenetic memory exists in this species.

Strawberry species

Fragaria vesca, also known as woodland strawberry, is common throughout Eurasia. It has entomophilous flowers, is short in height, perennial, herbaceous, and able to reproduce both asexually and sexually (Johnson et al., 2014). The genus *Fragaria* consists of 13 diploid species, four tetraploid species, one hexaploid species, and four octoploid species (Folta and Davis, 2006). Evidence suggests that *F. vesca* has been grown in European gardens for over 1000 years (Wilhelm and Sagen). The cultivated octoploid strawberry, *Fragaria* × *ananassa* (2n = 8x = 56), is a relatively young species that was derived around 300 years ago (Darrow, 1966). *Fragaria vesca* is believed to be a distant ancestor of *Fragaria* × *ananassa*. Thus, *F. vesca* is used as a reference to better understand the genomics of the octoploid *F.* × *ananassa* (Potter et al., 2000; Shulaev et al., 2008; Liston et al., 2014). Another reason for using *F. vesca* as a model plant of *F.* × *ananassa* is that its genome size is only 219 Mb divided across seven chromosomes and that its genome shows a high level of collinearity to *F.* × *ananassa* (Edger et al., 2018; Rousseau-Gueutin et al., 2008). *Fragaria vesca* has a very wide geographical distribution and grows from southern European regions with hot and dry summers (latitude 37°N) up to arctic conditions in Norway (latitude 70°N). *Fragaria vesca* has two reproduction systems: sexual reproduction with production of achenes and asexual reproduction through stolon formation. Because both reproductive systems have a relatively short generation cycle, *F. vesca* is an excellent model system for studying climatic responses in perennial plants.

DNA methylation is a major epigenetic regulatory mechanism

DNA methylation refers to cytosine bases in DNA modified by a methyl group, 5′-Methylcytosine. Early work emphasized a special role of 5′-Methylcytosine in nucleic acids (Sinsheimer, 1955). In 1979, Clear et al. found that C in CCGG sequence from different eukaryotes organisms is methylated using the restriction enzyme Mspl for digestion (Stein et al., 1983). Digestion using restriction enzymes, like Hpa II (CCGG) and Hha I (GCGC), which have a CpG in their recognizing site, are inhibited by methylation of C (van der Ploeg and Flavell, 1980; Razin et al., 2012). Cytosine methylation is considered a very important regulatory mechanism on the DNA level and occurs in both prokaryotes and eukaryotes. Early research revealed that gene expression might correlate to DNA methylation status (Bird et al., 1985; Laurent et al., 2010). In comparison to mammals, where most cytosine methylation occur in the CG context, plants have relatively loose methylation principle, including the DNA contexts CG, CHG (H refers to A, T, or C), which are symmetric sequences, and CHH, which

is an asymmetric sequence (Law and Jacobsen, 2010). Centromeric regions and some other genome regions that contain repetitive DNA, like satellite DNA and the intragenic regions of some genes, have high-level methylation (Zhang et al., 2006; Weber and Schübeler, 2007).

In eukaryotes, two DNA methylation mechanisms are needed: one is de novo methylation, and the other is maintenance methylation at hemimethylated DNA after DNA replication. These two types of DNA methylation are both catalyzed by DNA-methyltransferases (Tirot et al., 2022). In plants, there are also 3 types of DNA-methyltransferases: METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3), and DOMAINS REARRANGED METHYLTRANSFERASE (DRM2). Arabidopsis has four MET homologs (MET1, MET2 and b, MET3), three CMT homologs (CMT1, 2, 3) and three DRM homologs (DRM1, 2, 3) (Tirot et al., 2022). MET1 functions as a maintainer of symmetric CG methylation (Kankel et al., 2003). The role of MET2a and 2b are currently unknown (Jullien et al., 2012). MET3 is regulated by polycomb complexes in endosperm development (Tirot et al., 2022). CMT3 is plant specific and is required for the maintenance of CHG methylation (Cao et al., 2003). CMT2 methylates CHH when DECREASE IN DNA METHYLATION I (DDM1) mediates (Zemach et al., 2013). CMT1 mainly exists in reproductive tissue (Klepikova et al., 2016). DRM2 takes charge of de novo DNA maintenance. Plants also possess another plant-unique DNA methylation mechanism—RNA directed DNA methylation (RdDM). DRM2 is regulated by the RNA-directed DNA methylation pathway (Lindroth et al., 2001; Cao et al., 2003; Pontes et al., 2006). DRM1 can be detected in reproductive tissue (Jullien et al., 2012)

DNA methylation in plants

Overview of DNA methylation in plants

RNA-directed DNA methylation (RdDM) is unique to plants. RdDM depends on two RNA polymerases that are only found in plants: RNA POLYMERASE IV (Pol IV), and RNA POLYMERASE V (Pol V) (Haag and Pikaard, 2011; Eun et al., 2012). Pol IV transcribes at target loci and an RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) forms double-stranded RNA that is diced by DICER LIKE 3 (DCL3) into 24 nt small interfering RNAs (siRNAs). These siRNA are then exported to the cytoplasm and methylated by HUA ENHANCE 1 (HEN1). Methylated siRNA binds to ARGONAUTE 4 (AGO4) and then the siRNA-AGO4 complex returns into the nucleus (Figure 1). Together with Pol V, the complex recruits DNA methyltransferases to de novo methylate target sites (Ji and Chen, 2012; Pikaard et al., 2012). Histone modification could also participate in RdDM. SAWADEE HOMEODOMAIN

HOMOLOGUE 1 (SHH1) joins the complex and and allows the recognition of the methylated H3K9 and demethylated H3K4 (Law et al., 2011; Haag et al., 2012; Zhang et al., 2013; Law et al., 2013). A SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5-like) protein is also reported as a member of the RdDM complex. It can significantly reduce the methylation status of a retrotransposon AtSN1 and 5S rRNA when it mutates. The accumulation of 24 nt siRNA in its mutant suggests that it might act as an elongation factor for Pol V (Wang and Dennis, 2009). Transcripts generated by Pol V usually don't contain classic mRNA structure—5’ caps or poly A tails. Those transcripts only interact with siRNA within the scaffold RNA structure (Wierzbicki et al., 2008). Although RdDM methylation can occur at a wide range of genome location, it does not take place around pericentromeric region or heterochromatin. In these areas, only the other methylation mechanisms happen (Schoft et al., 2009; Zemach et al., 2013).

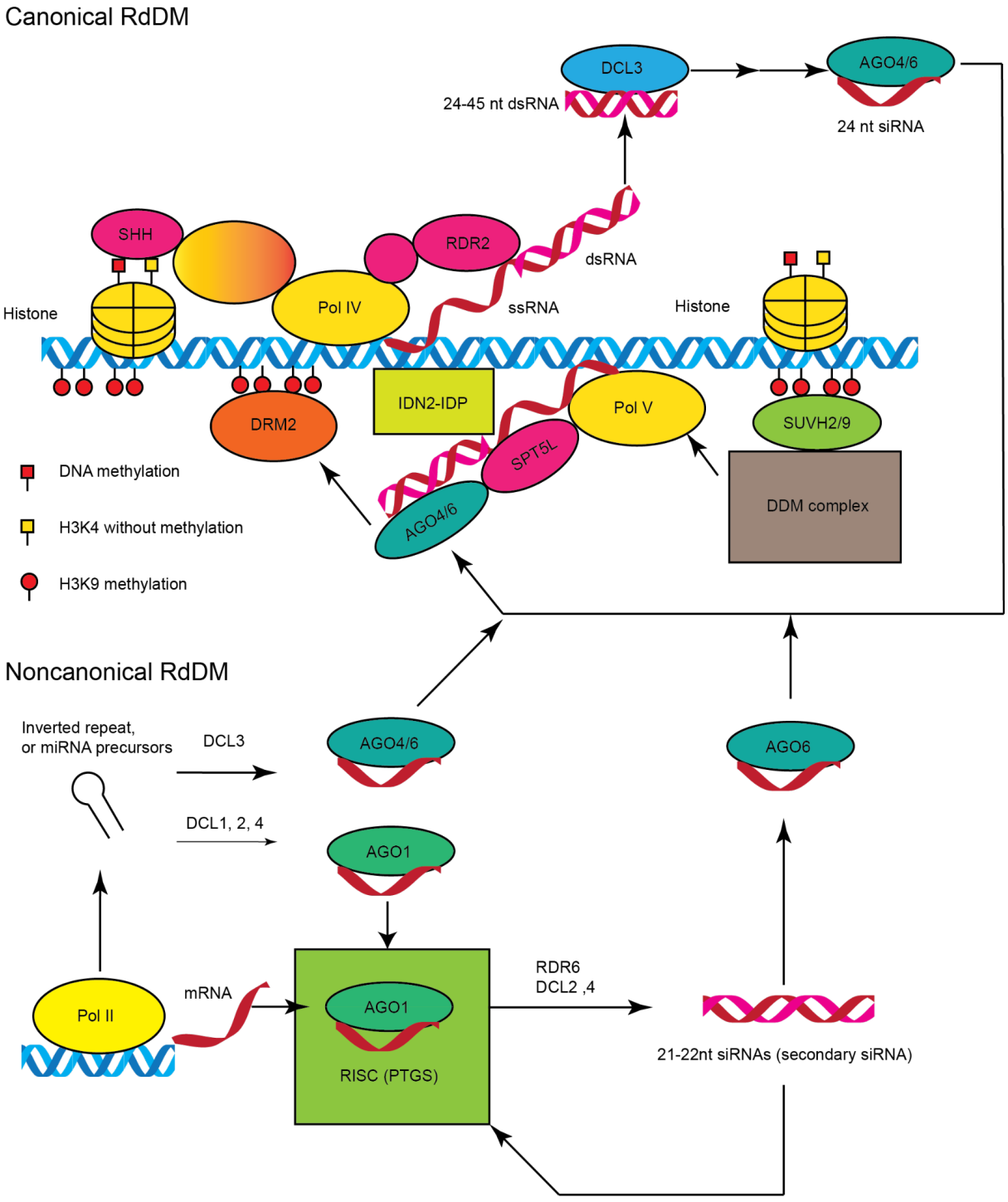

FIGURE 1. Schematic overview of the canonical RdDM pathway (top) and the non-canonical RdDM and RNAi/PTGS pathway (bottom). The canonical RdDM pathway can be broken into (1) sRNA production and (2) targeting DNA methylation to sites of sRNA production. The non-canonical RdDM pathway is closely related to RNAi and other PTGS pathways, and differs from canonical RdDM primarily in the source of sRNAs and sRNA processing. H3K9 = lysine 9 on histone H3; H3K4 = lysine 4 on histone H3; ssRNA = single-stranded RNA; dsRNA = double-stranded RNA, miRNA = microRNA. (Adapted from Erdmann and Picard, 2020)

Besides canonical RdDM, noncanonical RdDM pathways also exist. As Pol IV transcripts, the transcripts from Pol II can also enter RdDM after dicing by DICER LIKE 3 (DCL3) (Panda et

al., 2016). These Pol II transcripts could be from microRNA genes or stem from transcription of inverted repeat regions. In a transposon- specific RdDM mechanism, called RDR6-dependent RdDM, some newly formed transposons could be transcribed by Pol II. Those transcripts can be used as templates to synthesize dsRNAs by RNA DEPENDENT RNA POLYMERASE 6 (RDR6) (Allen et al., 2005; Panda et al., 2016). The dsRNAs can trigger and enter not only the RNA- silencing pathway to generate 21-22nt siRNA, but also the RdDM pathway with AGO2, Pol V and DRM2 (Nuthikattu et al., 2013). The siRNA biosynthesized through the RdDM pathway will guide a protein complex to methylate transposon sequences and enhance Post-Transcriptional Gene Silencing (PTGS). A variation of this RDR6-dependent RdDM event can be applied to newly integrated retrotransposons (Marí-Ordóñez et al., 2013). A protein, NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD) can mediate the bind of AGO2 and Pol IV and V and guide the complex to chromatin to inhibit histone modification and start RdDM (Pontier et al., 2012).

In plants, high DNA methylation level frequently comes up with transposable elements (Zhang et al., 2006). Thus, mutants with methyltransferase deficient or DNA methylation-related protein deficiency usually suffer from reduced silencing of transposable elements and leads to massive transposon activation, as exemplified by mutation of the chromatin remodeler DECREASE IN DNA METHYLATION I (DDM1) (Law and Jacobsen, 2010). The functional loss of DDM1 also causes the CACTA family transposon to activate and transpose to new sites (Kinoshita et al., 2004). Similarly, the deficiency of DNA maintenance methyltransferase MET1 will result in the evocation of a long terminal repeat (LTR) transposon EVD (Tsukahara et al., 2009).

Genomic imprinting is another mechanism which could be influenced by DNA methylation. The maternally imprinted gene FLOWERING WAGENINGEN (FWA) is maternally silenced in wild type plants through hyper methylation of its promoter region. FWA is only expressed from the maternal allele in the endosperm of the seed (Kinoshita et al. 2004). However, in its epigenetics mutant, the paternal expression of FWA is activated (Kinoshita et al. 2004). The FWA imprinting requires DEMETER (DME), which is only expressed during in the central cell of the female gametophyte, giving rise to the endosperm after fertilization. Thus, DME removing methyl markers could cause maternal expression of the imprinted gene (Kinoshita et al., 2007; Hsieh et al., 2009; Gehring et al., 2009). Due to this feature of DME, TEs could have their repressive DNA methylation marker expressed in endosperms, and new imprinted genes could be released from DNA methylation. Hence, a hypothesis indicates that methylation of

TEs which insert at the neighborhood region of the gene might be the basis of imprinting in plants (Gehring et al., 2009; Zemach et al., 2013). siRNA which could be involved in RdDM was found to be highly expressed in early embryo development. Furthermore, these siRNAs originate from female gamete and continue to persist even in immature seeds. This suggests that the gene encoding this siRNA may also be imprinted (Mosher et al., 2009). The male gametophyte, the pollen, contains two sperm and one vegetative cell. The perm and the vegetative cell have a totally different DNA methylation pattern: The vegetative cell is hypomethylated compared to sperm cells. Transposable elements expressed by demethylation in the vegetative nucleus may be very essential for the suppression of the same targets in sperm cells. 21nt siRNA synthesis from transposable elements (TEs) in the vegetative cell could move to sperms to silence the TEs by post transcriptional gene silencing (PTGS) (Slotkin et al., 2009).

DNA demethylation

Methylated DNA can be reversed into an unmethylated state by two mechanisms: passive demethylation and base excision repair-coupled demethylation. Passive demethylation is a relatively simple mechanism: without new methylation DNA replication will over time dilute old methylated DNA. Active demethylation, on the other hand, is more complex and differs between plants and animals.

In plants, methylated cytosines in DNA are replaced by unmethylated cytosines by DNA glycosylase and base excision repair (Ikeda and Kinoshita, 2009; Zhu, 2009). There are four bifunctional 5-methylcytosine glycosylases in plants: REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DME-LIKE 2 (DML2), and DML3 (Penterman et al., 2007; Ortega-Galisteo et al., 2008). These glycosylases can remove methylated cytosine from the double stranded DNA and leave behind a pyrimidine. Then ROS1/DME excise the DNA backbone at the pyrimidine site (Agius et al., 2006; Morales-Ruiz et al., 2006a), base excision-repair takes over the whole process and excises adjacent nucleotides of the 3’ end and fills the gap by synthesizing new DNA. ROS3 is another essential component of DNA demethylation. It can act as a regulator of DNA methylation to prevent over-methylation. It binds to single stranded RNA (ssRNA) and guides ROS1-mediated DNA demethylation (Zheng et al., 2008).

DNA methylation, histone modification, and heterochromatin

As one of the major epigenetic regulatory mechanisms, DNA methylation is not always independent from other mechanisms. The key points of crosstalk between DNA methylation and histone modifications are “reader” proteins, for example the histone modification readers

PLANT HOMEODOMAINS (PHD), chromodomains and bromo adjacent homology domains (BAH), and the DNA methylation readers of DNA methylation, like the SET- and RING-associated (SRA) domain, CXXC domain and methyl-CpG- binding domains (Du et al., 2015).

In Arabidopsis, KRYPTONITE (KYP) proteins have activities of recognizing DNA methylation and methylates H3K9me (Jackson et al., 2002; Johnson et al., 2007). Global CMT2- and CMT3-dependent DNA methylation requires KYP and its homologs SU(VAR)3-9 HOMOLOG 5/6 (SUVH5/6)) (Zemach et al., 2013). Research also shows that CMT2, CMT3, DOMAINS REARRANGED DNA METHYLASE 1 (DRM1), and DRM2 are involved in H3K9 methylation (Stroud et al., 2014). SUVH9 and its homolog, SUVH2, are key components of the required for RdDM, and are recruited to methylated DNA, but they don't have DNA methyltransferase activity (Johnson et al., 2008; Liu et al., 2014). The histone demethylase LYSINESPECIFIC DEMETHYLASE LIKE 1 (LDL1) and LDL2 could remove H3K4me2 and H3K4me3 to allow SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1) binding and starting the generating of RNA POLYMERASE IV (Pol IV) related small interfering RNA (siRNAs) (Greenberg et al., 2013). HISTONE DEACETYLASE 6 (HDA6) also has a similar function by deacetylating histone to initiate Pol IV and siRNA synthesis (Aufsatz et al., 2002, 2007).

In mammals, there is a strong correlation between DNA methylation and certain types of histone modifications (Mikkelsen et al., 2007; Meissner et al., 2008). The methylation of H3K9 can be mediated by the SUV39 protein family which contains SET domain (Rea et al., 2000; Schultz et al., 2002). SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1) could tri-methylate H3K9 at the genome region around endogenous retrovirus (ERVs) and inactivate the X chromosome (Karimi et al., 2011; Minkovsky et al., 2014). At active promoters, binding of transcription factors will prevent methylation of CpGs, and H3K4 methylation at the region nearby would also protect CpGs from methylation (Smith and Meissner, 2013). The ADD domain of *de novo* methyltransferase, (CYTOSINE-5)-METHYLTRANSFERASE (DNMT3s), could recognize unmodified H3 and can be repressed by H3K4me (Edwards et al., 2010; Eustermann et al., 2011). PWWP domain recognizing H3K36me3 could be also influenced by the correlation between RNA polymerase II and DNMT3B (Jin et al., 2012; Morselli et al., 2015)

Epigenetic memory

Different types of epigenetics memory

Transgenerational inheritance of epigenetic modifications is still controversial in some circles because of a mechanism which erases epigenetics marks of the whole genome during meiosis and early gametogenesis (as is the case in mammals and some other organisms). However, at least some genetic modification can be inherited to the progeny through mitosis (asexual) and even meiosis.

Xu et al. constructed a stable hypomethylated epigenetic population (propagated mitotically and meiotically) in woodland strawberry by using DNA demethylation reagent 5- azacytidine (5-azaC) before selection the extreme phenotypes for further study (Xu et al., 2016b, 2016a). The flowering time changes are believed to be caused by methylation changes of CG or CHG sites in the early flowering populations. Their work has proved that epigenetic marks, especially DNA methylation, could be subject to selection (Xu et al., 2016b, 2016a). In their early flowering populations, they found a FLOWERING LOCUS T (FT), a NUCLEAR FACTOR Y, SUBUNIT B2 (NFYB2), a FRUITFULL (FUL), a SEPALLATA3 (SEP3) and other genes downstream of flowering regulatory pathway were upregulated and believed as contributor to flowering change. Notably, the CG(CGN) methylation in the FT gene had significant change (Xu, 2016).

Latzel and Münzbergová (2018) allowed *F. vesca* plants only to respond to certain soil nutrients under particular light intensity. In the following generation, progeny was given the same light intensity without soil nutrients. These plants had similar biomass as their parent even without nutrients (Latzel and Münzbergová, 2018). Recently it has been proposed that DNA methylation can mediate local adaptation and response to climate change in *Fragaria vesca* (Sammarco et al., 2022).

Also, when Arabidopsis faces high osmotic stress, the plants induce some form of epigenetic memory to increase plasticity of the phenotypes and pass this kind of ‘epigenetic memory’ to the next generation but mainly maternally. This kind of memory is mainly caused by DNA methylation changes, and can be reinforced by repeated suffering of the stress but fade when the stress disappears (Wibowo et al., 2016). Not only stress-related ‘epigenetic memory’ is reported to be inherited by the next generation, but also phenotypic impacting epimutants. A very well-known epimutation was discovered in species toadflax already in 1742, described by Carl Linnaeus in 1744 (Gustafsson, 1979). This epimutation causes toadflax to have five spurs

rather than four, the normal spur number. The mechanism of this epimutation was identified as the silencing caused by high DNA methylation in a homologue of the cycloidea gene, Lcyc, in 1999 (Cubas et al., 1999). When a line with a low-methylation genome crosses with a normal wild type line in *Arabidopsis thaliana*, massive methylation changes, and expression changes happen in the next generation progeny. Many loci show distinct and inherited epialleles from parents, whereas transposon in pericentromeric region being the most affected by the methylation change (Rigal et al., 2016). Moreover, DMRs act like genetic traits co-segregating with genetic background in some recombinant inbred lines (RILs) in both soybean and *Arabidopsis* (Johannes et al., 2009; Nery et al., 2013). The flowering time and height changes epigenetic Recombinant Inbred Lines (EpiRILs) in *Arabidopsis* could last 8 generations (Johannes et al., 2009). The EpiRILs constructed in DECREASE DNA METHYLATION 1 (DDM1) deficient background change in branching leaf area correlate with altered epigenetic regulation (Kooke et al., 2015). More intriguingly, organ-specific methylation markers inherited by asexual offsprings and maintained even following meiosis, induced the phenotypic changes that depends on tissue origin. Clonal populations with different tissue origin respond differently under biotic stress (Wibowo et al., 2018).

In the long-lived gymnosperm Norway spruce, increased temperature sum during the embryogenesis stage induces epigenetics memory affecting the timing of bud burst and bud set in progenies (epitypes) in a predictable and reproducible manner even though plants are genetically identical (Kvaalen and Johnsen, 2008; Carneros et al., 2017; Yakovlev et al., 2010, 2014, 2016). Transcriptome analysis discovered that 35 of 448 transcripts from genes having epigenetic functions are differentially expressed between such epitypes (Yakovlev et al., 2014). Furthermore, a significant number of epigenetic regulators are differentially expressed during embryogenesis at different epitype-inducing conditions, supporting that methylation of DNA and histones, as well as sRNAs, are needed for the establishment of the epigenetic memory in Norway spruce (*Picea abies*) (Yakovlev et al., 2016). Further transcriptional profiling showed over 10000 genes differentially expressed. Among 735 putative epigenetic-related orthologs, 329 of them changed the expression levels due to epitype inducing temperature conditions (Yakovlev et al., 2016). Besides epigenetic-related genes, gene expression analysis also showed that most DHNs (dehydrins), EARLY BUD BREAK 1 (EBB), and FLOWERING LOCUS T LIKE 2 (FTL2) genes had changed their transcription level between eiptypes before or during bud burst (Carneros et al., 2017). Also, 7 conserved and 9 novel miRNAs were differentially

expressed between epitypes in the bud set, and a putative target study revealed that they might participate in epigenetic regulation (Yakovlev et al., 2010).

Classical vernalization

The most well studied Epigenetic memory response to temperature is Vernalization. Vernalization refers to a process of promoting flowering time in some plants by low temperature treatment (winter). Trofim Lysenko worked on cold treatment on cereal seeds, and coined the term “jarovization” (vernalization) in 1928 to describe a chilling process he used to treat seeds of winter cereals to behave like spring cereals. The formal definition of vernalization "the acquisition or acceleration of the ability to flower by a chilling treatment" was first given by botanist P. Chouard in 1960 (Chouard, 1960). The most famous gene module in the vernalization process is FLOWERING LOCUS C (FLC), a MADS box domain containing flowering suppressor, in *Arabidopsis thaliana*. The expression level of FLC is regulated by RNA POLYMERASE-ASSOCIATED FACTOR 1 COMPLEX (Paf1C) and FRIGIDA (FRI) in the reproduction and embryo stages. Paf1C is a elongator of RNA polymerase II transcription. FRI promotes the transcription of FLC together with a WD40 REPEAT protein (ATWDR5a), histone methylases (ARABIDOPSIS TRITHORAX-RELATED7 (ATXR7) and ARABIDOPSIS HOMOLOG OF TRITHORAX (ATX1 and ATX2)) and deposition of histone variant (H2A.Z). FRI forms a super complex with COMPASS-like (H3K4 methyltransferase complex) which can alter the chromatin status near FLC, change the spatial structure of chromosomal region nearby to further activate the transcription and promote faster elongation and alternative splicing (Michaels et al., 2004; Schmitz et al., 2005; Kim and Michaels, 2006; Andersson et al., 2008; Choi et al., 2011; Li et al., 2018). One of the histone mark changes is the demethylation of histone 3 lysine 27 (H3K27me3). The tri-methylation of H3K27 is done by a polycomb repression complex (PRC2) consisted by VERNALIZATION 2 (VRN2), SWINGER (SWN, an E(z) histone methyltransferase homologue), FERTILIZATION-INDEPENDENT ENDOSPERM (FIE, an extra sex combs (ESC) homologue) and MUSASHI RNA BINDING PROTEIN 1 (MSI1, a p55 homologue) (Jiang et al., 2009; Ko et al., 2010; Li et al., 2018). Other histone modifications also involved in the regulation of FLC, such as histone acetylation, the methylation of H3K36 and monoubiquitination of H2B (Zhao et al., 2005; Gu et al., 2009; Jiang et al., 2009; Ko et al., 2010; Li et al., 2018). FLC can be silenced by low temperature, and it has been demonstrated that a group of non-coding RNA (ncRNA), COLD INDUCED LONG ANTISENSE INTRAGENIC RNA (COOLAIR), COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR) and COLD OF WINTER-INDUCED

NONCODING RNA FROM THE PROMOTER (COLDWRAP) participate in this silencing process (Swiezewski et al., 2009; Heo and Sung, 2011; Kim and Sung, 2017). COOLAIR is an antisense transcript started from the poly-A signal of FLC. The FLC terminator and COOLAIR promoter are very essential for ncRNA's transcription. Unlike COOLAIR, both COLDAIR and COLDWARP are the non-coding RNAs from the sense transcripts without poly-A signal and splicing of introns. COLDAIR and COLDWRAP are transcribed directly from the first intron of FLC and initiates their transcription later than COOLAIR (Kim and Sung, 2017). COLDAIR is believed to guide the histone methyltransferase subunit (CLF) of PRC2 to the FLC site (Kim et al., 2017). Besides COOLAIR, COLDAIR, and COLDWRAP, another non-coding RNA, MADS AFFECTING FLOWERING 4 (MAF4) antisense RNA can also be induced by cold, and can promote the expression of MAF4 (Zhao et al., 2018). PRC2 can bind to the histone near FLC before low temperature treatment, and recruit PLANT HOMEODOMAIN PROTEINS (PHD) after low temperature induction. The PHD-PRC2 complex forms at the second intron of FLC and covers the part of the intron that is believed to be the start of COLDAIR. Furthermore, 2 circadian clock genes *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL1* (*LHY1*) have been shown to be able to sense cold and to bind to the specific elements called the evening elements in the promoter region of VERNALIZATION INSENSITIVE 3 (VIN3) to prompt the expression level of VIN3 (Kyung et al., 2021). But this kind of histone mark transition will not prohibit the FLC in mature leaves. VRN1, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) and Arabidopsis PROTEIN ARGININE METHYLTRANSFERASE 5 (ATPRMT5) are believe participate the silencing process of FLC after putting back to normal condition. LHP1 could recognize the H3K27me3, could bind to a nucleation region, that one intronic single nucleotide change at the 3′-end of it, with a cis element called COLD MEMORY ELEMENT (CME) with VP1/ABI3-LIKE 1/2 (VAL1/2) (Yuan et al., 2016; Costa and Dean, 2019).

The vernalization status must be reset in the progenies to make sure the next generation will not flower without winter treatment or vernalization. The expression of FLC is suppressed in gametes cells but is promoted again after fertilization by LEAFY COTYLEDON1 (LEC1), which binds to the promoter region of FLC and increases H3K4me3 and H3K36me3 levels (Tao et al., 2017). Two other plant specific proteins LEAFY COTYLEDON2 (LEC2) and FUSCA3 (FUS3) can obstruct the binding of the PHD-PRC2 complex and reset chromatin near FLC to the active state (Figure 2). This could also lead to the expression of FLC in the embryo stage (Li et al., 2018; Tao et al., 2019). When germinated seedlings have vernalization

treatment, PHD-PRC2 complex binds to the CME to methylate histone 3 lysine 27 with the effect that VAL1/2 prevents the expression of FLC (Tao et al., 2019).

FLC is involved in complex regulatory networks that require genetic and epigenetic regulation and transcriptional and post transcriptional regulation, including DNA methylation, histone methylation and demethylation and long non-coding RNA. Strikingly, this complex mechanism is not conserved in all plants. Some plants don't have FLC, e.g., *Rosaceae*, but still have vernalization.

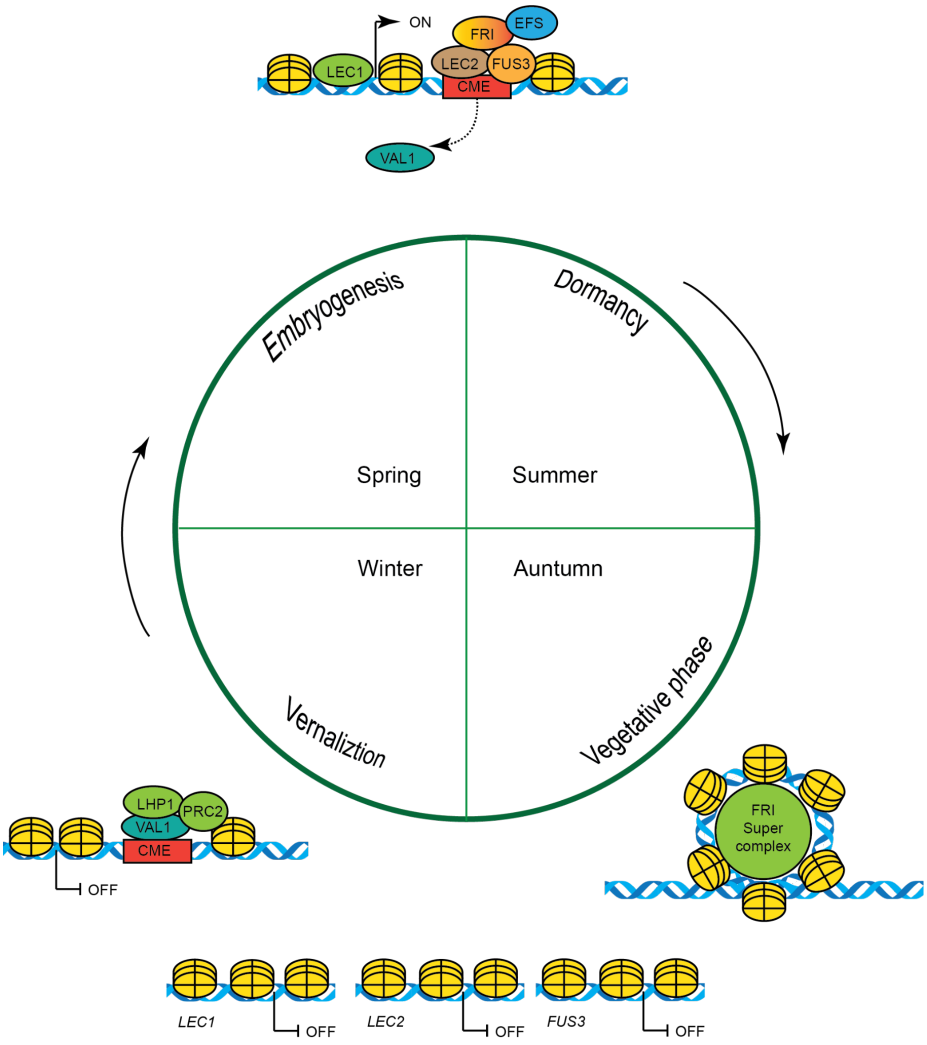

FIGURE 2. Molecular epigenetic regulation of *FLC* expression in response to developmental and seasonal changes in *Arabidopsis* winter annuals (Adapted from Luo and He, 2020)

Vernalization in *Fragaria vesca* and Rosaceae

Fragaria species can be vernalized by 5-weeks of 1 to 4 ºC treatment (Chouard, 1960). Heide and (Heide and Sønsteby, 2007) found that a cold exposure promotes *F. vesca* flower formation. Unlike all other populations they studied, a high-latitude population from 70°N had an obligatory vernalization requirement (Heide and Sønsteby, 2007). Perpetual flowering strawberries are day-neutral under a condition of 9 °C (Bradford et al., 2010). Non-dormant raspberries need chilling at 6°C for several weeks to vernalize (Sønsteby and Heide, 2009). A low temperature is needed to terminate dormancy to flower in apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis* L.) (Heide and Prestrud, 2005). Seasonal flowering *Rosa*×*wichurana* might need vernalization to form flowers next spring (Foucher et al., 2008). In diploid strawberry *Fragaria vesca*, the vernalization process involves one essential gene, TERMINAL FLOWER 1 (FvTFL1). FvTFL1 is an FT-independent flowering time repressor which responds to the photoperiod length. The expression of FvTFL1 could be suppressed by low temperature, but activated by SUPPRESSOR OF CONSTANTS (FvSOC1) under the LD condition (Koskela et al., 2012, 2017; Mouhu et al., 2013). FvSOC1 was found as conjunction of flowering regulation pathway and gibberellin (GA) biosynthesis by regulated by FLOWERING LOCUS T 1 (FvFT1) and activating genes in GA biosynthesis pathway (Mouhu et al., 2013). Studies in apple and pear unveiled that silencing the expression of TFL homologs could lead to early flowering (Freiman et al., 2012; Flachowsky et al.). Evidence from researches in *F. vesca*, apple and pear might point to the fact that TFL homologs in *Rosaceae* could play the most important role in vernalization (Kurokura et al., 2013).

Vernalization in the annual plant wheat

In wheat (*Triticum aestivum*), the vernalization process is mainly controlled by a few genes, TaVRN1(*VERNALIZATION 1*), TaVRN2, TaVRN3, and TaVER2 (VERNALIZATION RELATED 2). Among them, TaVRN1 was identified as a FRUITFUL (FUL) like MADS box-containing transcription factor at the central position of vernalization regulation (Yan et al., 2003). TaVRN2, a zinc finger transcription factor, together with VEGETATIVE TO REPRODUCTIVE TRANSITION 2 (TaVRT2), a SHORT VEGETATIVE PHASE (SVP) - like MADS box transcription factor, repress the expression of TaVRN1(Yan et al., 2004; Karsai et al., 2005; Szűcs et al., 2007; Diallo et al., 2010). TaVRT2 binds to CArG motif of the TaVRN1 promoter (Kane et al., 2007). Both TaVRN1 and TaVRT2 are produced in leaves during vernalization (Xie et al., 2021). Ta VRN1 ALTERNATIVE SPLICING (VAS), a non-coding RNA from sense transcript of TaVRN1 only found in winter wheat, can also regulate

the expression of TaVRN1 through association with TaRF2a-TaRF2b (a heterodimer of basic leucine zipper transcription factors). The complex binds the promoter region of TaVRN1 during the middle period of vernalization to recognize the sp1 motif in the promoter (Xu et al., 2021). Furthermore, the expression level of TaVRN2 is gradually reduced in the vernalization and remains repressed after vernalization to initialize the photoperiodic flowering time regulation. Photoperiodic flowering time is mediated by TaVRN3 (TaFT1), a FLOWERING LOCUS T homolog in winter wheat (Chen and Dubcovsky, 2012; Oliver et al., 2013; Shimada et al., 2009; Deng et al., 2015). VER2, a jacalin lectin, directly recognizes the pre-mRNA of TaVRN1 together with another GLYCINE-RICH RNA-BINDING PROTEIN, TaGRP2. TaGRP2 represses the mRNA level of TaVRN1. O-GlcNAc modification of TaGRP2 protein can obstruct the recognition of TaVER2 and associated with O-GlcNAc modification to suppress the level of TaGRP2 protein (Xiao et al., 2014).

Just like for *Arabidopsis thaliana*, the vernalization machinery in Wheat is related to chromatin modification. The H3K27me3 and H3K4me4 and histone methyltransferases and methyltransferases complexes (PHD-PRC2 and COMPASS-like) play an important role in vernalization (Oliver et al., 2009; Mozgova and Hennig, 2015; He and Li, 2018).

Vernalization in perennial Arabis and Arabidopsis plants

In *Arabidopsis lyrata*, a perennial relative of *Arabidopsis thaliana*, only FLC1 and FRIGIDA homologs have shown competence of regulating vernalization. In another perennial model in Brassicaceae, *Arabis alpina*, PERPETUAL FLOWERING 1 (PEP1), the ortholog of FLC, a SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 (SPL15), PEP2 (AP2 transcription factor), miR156, miR172, SOC1, LEAFY (LFY1), TFL1 and APETALA1 might participates in vernalization (Lazaro et al., 2018; Tilmes et al., 2019; Hyun et al., 2019; Bergonzi et al., 2013; Lazaro et al., 2019; Wang et al., 2011).

Aim of the study

Global warming is a serious challenge to plants' ability to adapt to a rapid changing environment. In quantitative genetics, the phenotypic variation results only from the genotype and environmental variation and their interactions. However, epigenetic changes also contribute to phenotypic diversity and this fact has led to a reexamination of the underlying nature of environmental caused variance (Hirsch, Baumberger & Grossniklaus 2012; Diez, Roessler & Gaut 2014).

Epigenetics is the study of heritable phenotype changes that do not involve a change in the nucleotide sequence of the genome. Epigenetics involve changes that affect gene expression but is in principle used to describe any heritable phenotypic change (Dwivedi, Herman, McCaffrey & Raj 2011; Feng & Jacobsen 2011). Such effects on cellular and physiological phenotypic traits can result from external factors such as changes in environmental conditions. When the epigenetically induced change due to changes in the environment (temperature, drought or the like) impact important phenological traits, and create genetically identical but phenotypically distinct individuals (epitypes) that show an adaptive response, we call this phenomenon epigenetic memory (Carneros et al. 2017).

Thus, in the context of climate change, plants´ adaptation to abiotic cues is crucial for their performance and species survival (i.e., fitness). Increasing temperatures is a major environmental challenge that plants have to face. Phenotypic plasticity such as that generated by epigenetic changes is one of the strategies they can use to resist and thrive under its effects (Nicotra et al. 2010). Some species may possess adaptation mechanisms that counteract at least part of the effects from increasing temperatures. The long-lived gymnosperm *Picea abies* can adjust to the temperature conditions during sexual and asexual reproduction by generating epitypes with altered bud phenology, in a predictable and reproducible manner, having an epigenetic memory of the environmental condition experienced during embryogenesis (Kvaalen & Johnsen 2008). Such a memory represents an epigenetic mechanism to increase the phenotypic plasticity to climate change, but it is not yet known how common a feature this type of temperature memory is among plants in general. However, one of the most famous and well-understood epigenetic memory-like impacts of temperature, is vernalization, the memory of low temperatures (winter) that induces some plants to flower. The epigenetic mechanism behind vernalization, with the gene FLC at its core, is very well described in the annual angiosperm *Arabidopsis thaliana*. However, *Arabidopsis thaliana* is an annual plant, and its life cycle is

very different from the long-lived perennial plants, making it a less than ideal model to study epigenetic memory phenomena in perennial plants such as long-lived trees and the model for the Rosaceae-family of plants *Fragaria vesca*.

Based on this prior knowledge about epigenetic memory responses to temperature, the main goal of this PhD thesis was to investigate whether an epigenetic memory to the temperature conditions during asexual and sexual reproduction exist in European ecotypes of the perennial angiosperm *Fragaria vesca.*

To this end we aimed to investigate if different temperature conditions during both asexual and sexual reproduction induced lasting phenological changes that could be tested under common garden conditions. A sub-aim was to uncover differences in epigenetic memory plasticity among the ecotypes and between the two modes of reproduction.

We also aimed at studying the impact of the treatments on the transcriptome and what members of the epigenetic machinery and other important pathways are affected.

We also aimed to study the DNA methylome changes and if these were related to the transcriptome changes and to the predicted induced phenological change (indicative of an epigenetic memory response) to the temperature treatment in both modes of reproduction.

Results and Discussion

In this PhD research, I present our findings indicating that the temperature during sexual and asexual reproduction cause phenology changes, methylome changes (Papar I & II) and transcriptomic reprogramming (Paper III) indicative of epigenetic memory in some European *Fragaria vesca* ecotypes.

We used whole genome bisulfite sequencing, RNA sequencing and phenology observations to investigate the memory of temperature sum differences during asexual and sexual reproduction in the perennial plant, *Fragaria vesca*.

Phenotypes are changed in response to temperature treatments during asexual and sexual propagation

The ecotypes we used in our asexual and sexual experiment were collected from Norway (NOR2 and NOR29), Iceland (ICE2), Spain (ES12), and Italy (IT4) (Figure 3). They showed very different responses to the temperature sum experienced during propagation, in both asexual and sexual propagations, when the resulting plants were tested under common garden conditions.

FIGURE 3. Images of the European ecotypes used in this PhD research. Ecotypes: ES12 **(A)**, ICE2 **(B)**, IT4 **(C)**, NOR2 **(D)**, NOR29 **(E).** Scalebar, 10 cm.

To examine if the conditions during asexual reproduction affect the adaptive performance of the daughter plants, we treated four ecotypes of *Fragaria vesca* at two different temperatures only during the propagation period over three consecutive asexual generations. Phenology (flowering time, runner generation, and petiole length) was examined under common garden conditions for the resulting plants to identify putative epigenetic memory effects.

We found that the phenology of *Fragaria vesca* was impacted by temperature conditions during asexual reproduction, and this impact increased from the first to the third asexual generation. Flowering in high latitude ecotypes NOR2 and ICE2 was significantly altered in response to the treatment, indicative of an epigenetic memory response. However, the low latitude ecotypes IT4 and ES12 did not flower in the first generation and did not respond significantly to

treatment. The difference in time to flowering and high plasticity in NOR2 and ICE2 may be an adaptation to flowering in the shorter growth period at high latitude. The fact that the impact increase over three asexual generations suggests that the putative epigenetic effect observed on flowering time accumulates over several generations with repeated treatment.

The impact on stolons (ES12 and IT4) and petiole length (ES12, ICE2 and NOR2) was more marked in the first asexual generation, but remained significant for IT4 regarding stolon number and for petiole length in NOR2 after 3 asexual generations. Thus, a possible epigenetic impact of the treatment on these adaptive features is detectable, but is less convincing than that observed for flowering in the NOR2 and ICE2 ecotypes.

The impact of temperature during sexual propagation on phenotypes was also observed in some ecotypes. Elevated temperature during the embryogenesis phase promoted earlier flowering time in high latitude ecotype (NOR2) when tested under the common garden conditions. In addition to the difference in flowering time, NOR2 showed changes in petiole length and numbers of growth points. This could indicate that temperature treatment experienced during embryogenesis caused an epigenetic augmented phenotype temperature in NOR2. High plasticity in NOR2 may be an adaptation to flowering in the shorter growth periods at high latitude at its place of origin.

All phenological changes considered, the variable responses to the treatments in different ecotypes suggest that they differ in their epigenetic plasticity, NOR2 being the more plastic ecotype with significant response regarding both flowering time and petiole length in both asexual and sexual reproduction. However, the effects of temperature treatment during stolon formation and embryogenesis on phenotype are not directly comparable between different propagation methods for the following reason: 1. The length of treatment (temperature sum experienced) was not the same between reproduction methods. In asexual reproduction, the temperature treatment was reiterated for 3 generations. While during sexual reproduction, the temperature treatment was only applied to the embryogenesis stage after pollination. 2. Also the growth chamber conditions varied slightly between the two experimental setups. The asexual reproduction experiment was performed in two separate growth chambers for the two temperature conditions followed by growth in common garden in greenhouse at the University of Helsinki, whereas the pollination of sexual reproduction was carried out in the University of Helsinki in separate growth chambers for the two temperature conditions, but the germination

and growth under common garden conditions in the same growth chamber was carried out at the University of Oslo.

Methylome analysis following asexual and sexual propagation

Based on the phenotypic observations, we knew that at least some *F. vesca* ecotypes can response to treatment in both asexual and sexual reproduction. Ecotypes of *F. vesca* have evolved to their habitat. Thus, they may show different responses to environmental change, as well as, very different memory of the temperature conditions during asexual and sexual reproduction processes. However, the molecular mechanism such as possible importance of DNA methylation changes behind this induced response to contrasting temperature conditions during clonal (asexual) propagation and embryogenesis (sexual) propagation in these ecotypes must be investigated. .

A previous study in *F. vesca* suggested that the methylome provides the needed plasticity to plants and that population history is the key factor of shaping the methylome (De Kort et al., 2020). An analysis between temperate and tropic ecotypes of Lotus (*Nelumbo nucifera*) support that methylomes have impacts on transcriptomes and contribute to phenological plasticity (Li et al., 2021). Wibowo et.al reported that organ-specific epigenetic marks can be inherited partially through the asexual reproduction or even severals rounds of sexual propagation (Wibowo et al., 2018). They also discover that osmotic induced epigenetics alteration can be passed to the next generation through female gametes (Wibowo et al., 2016). Recent research revealed that CHH methylation located in the CmMYB6 promoter region could lead to color switching from pink to yellow, and could be inherited through clonal propagation in *Chrysanthemum morifolium* (Tang et al., 2022).

In order to decipher the genetic machinery behind the observed temperature memory in both asexual reproduction and sexual reproduction, we sequenced the methylomes and transcriptomes for all ecotypes and treatments.

Methylome of *Fragaria vesca*

In asexual reproduction, we used whole genome bisulfite sequencing to probe the methylomes of the ecotypes in response to treatment. Under normal conditions, the ecotypes displayed methylation differences over all chromosomes. ICE2 is generally hypermethylated, whereas NOR2 methylation is generally hypomethylated in comparison to the other ecotypes. Individual

hyper- and hypomethylated peaks occurred in all ecotypes, indicating that both global and specific methylation differences are numerous between ecotype methylomes. Having very different starting points regarding their methylomes we can expect to find both common and ecotype-specific methylome responses to treatment. A principal component analysis confirmed that the methylomes respond to treatment in a reproducible manner between biological replicates. The relative methylome induced changes are more pronounced for the CHH context than for the CHG and CGN contexts at 50kb resolution. That the ecotype differences are visible for all contexts in all ecotypes suggests that most ecotype specific induced methylation changes in CGN and CHG context occur at a higher level of resolution.

When comparing the induced methylation changes for all genes and repetitive elements, NOR2 shows the largest induced change. This suggests that NOR2 has a more plastic methylome than the other four ecotypes. Indeed, NOR2 showed significant induced changes in all genomic features (such as gene body, promoter, 3' region) in all methylation contexts. ICE2 lacked significant change for gene-body in the CHG context only, being the second most plastic methylome. In contrast to this, ES12 and IT4 responded poorly or not at all in genomic features in the CGN context. Repetitive elements and pseudogenes have similar levels of methylation both during normal conditions and similar increase in response to treatment in all ecotypes. The methylation induced in repetitive elements and pseudogenes was a significant response to treatment in all four ecotypes for all three methylation contexts. This is in contrast to the aforementioned genic features, where one or more ecotypes lacked significance in at least one genic feature for all contexts, suggesting that the effect at the gene level is more subtle or selective than for the REs and pseudogenes regarding what sites are differentially methylated. Similar results have been reported in mulberry (*Morus notabilis*) and *Arabidopsis* in response to pathogen treatment (Xin et al., 2021; Dowen et al., 2012).

In the asexual experiment, the predicted densities of different known genomic features (RE, genes and pseudogenes) were brought into context to explain their correlation to methylation through a linear regression model. This confirmed a negative correlation between DNA methylation and protein coding genes, and a positive correlation between DNA methylation and REs and to pseudo genes. However, only ~55% or less of the methylation can be explained by this linear regression model, hinting that half of the methylated cytosines are associated with other non-annotated genomic features not assessed by this model including long distance regulatory elements and chromatin conformation changes.

We found that the trinucleotide CCG in the CHG context is relatively hypomethylated and the trinucleotides CTA and CAA in the CHH context are relatively hypermethylated especially around the end of the Fvb1 chromosome and the middle of the Fvb3 and Fvb4 chromosomes. In *Arabidopsis*, maize, tomato, and rice, it was hypothesized that CCG methylation is maintained by both CMT3 and MET1 (Gouil and Baulcombe, 2016). MET1 first methylates CGG in the symmetric position. KRYPTONITE/SUVH4 (not SUVH5/6) then recognizes the CGG methylation and di-methylates H3K9me nearby. The di-methylation of H3K9 recruits CMT3 to methylated CCG. The relatively low methylation level of CCG is due to low efficiency of SUVH4 compared to SUVH5/6 (Gouil and Baulcombe, 2016). However, an explanation for the relative hypermethylation of CTA and CAA remains elusive.

For the sexual reproduction we applied the same strategy as with asexual reproduction. Whole genome bisulfite sequencing was performed to probe the methylomes of the ecotypes in response to temperature treatment. Under normal conditions, the ecotypes from achene displayed methylation differences over all chromosomes. ICE2 and NOR29 were generally hypermethylated, whereas NOR2 was hypomethylated in comparison to the other ecotypes. Individual hyper- and hypomethylated peaks occurred in all ecotypes, indicating that both global and specific methylation differences were numerous between seed derived ecotype methylomes. Again, we expected to find both common and ecotype-specific responses to treatment. The methylomes of all ecotypes responded significantly to the temperature treatment. That the ecotype differences were visible for the CGN and CHG contexts at 50Kbresolution, while also the induced responses to treatment were easily observable for CHH, for all ecotypes, suggest that most ecotype specific induced methylation changes in CGN and CHG context occur at a higher level of resolution.

When comparing the temperature induced methylation changes for pseudogenes and repetitive elements, NOR2 showed the most significant change in methylome. However, ICE2 had the most significant changes within genomic features. This suggests that ICE2, NOR2 and ES12 have the most plastic methylome of the ecotypes and mirrors the higher phenological plasticity observed in NOR2. In REs and pseudogenes, a significant response to temperature was found in all ecotypes for CGN methylation context except for NOR29.

Temperature-induced DMRs and DMGs

Here we define differentially methylated regions (DMRs) as regions with significant methylation changes. And we define differentially methylated genes (DMGs) as genes with DMRs located in its promoter or/and gene-body region. In the asexual experiment, not only did regions with low gene density tend to have more and larger differentially methylated regions (DMR) peaks, but also the less dense regions have numerous DMR rich chromosomal regions in all four ecotypes. NOR2 had the largest induced global methylation changes in response to the temperature treatment in all methylation contexts. Hence, the NOR2 ecotype has the most DMRs and differentially methylated genes (DMGs) compared to ICE2, ES12 and IT4.

The CHG and CHH DMRs were mostly located in the intergenic region and 3' direction of transcription start site (TSS), whereas the CGN DMRs occurred in the promoter, gene-body and 5' direction of TSS. In plants, CGN methylation is usually enriched in the gene-body region and depleted at the transcriptional start and termination sites. Typically, genes with high methylation in their body region are highly expressed and conserved (Tran et al., 2005; Takuno and Gaut, 2012, 2013; Seymour et al., 2014; Bewick et al., 2016; Bewick and Schmitz, 2017).

Regardless of ecotype, the most DMRs and DMGs were found in the CHG methylation context. In contrast to CGN methylation that shows both hypo- and hypermethylation, most treatment-induced CHG and CHH DMR´s were hypermethylated in all ecotypes (Figure 4). In plants, CHG and CHH methylation and maintenance are mediated by DNA methyltransferases CMT3 (CHG) and CMT2 and DRM1/DRM2 by RdDM (CHH) indicating the involvement of both pathways in response to treatment. This indicates more than one DNA methylation related pathway is triggered by the treatment. In plants, CHG and CHH methylation and maintenance are mediated by DNA methyltransferases CMT3 (CHG) and CMT2 and DRM1/DRM2 by RdDM (CHH) indicating the involvement of both pathways in response to treatment.

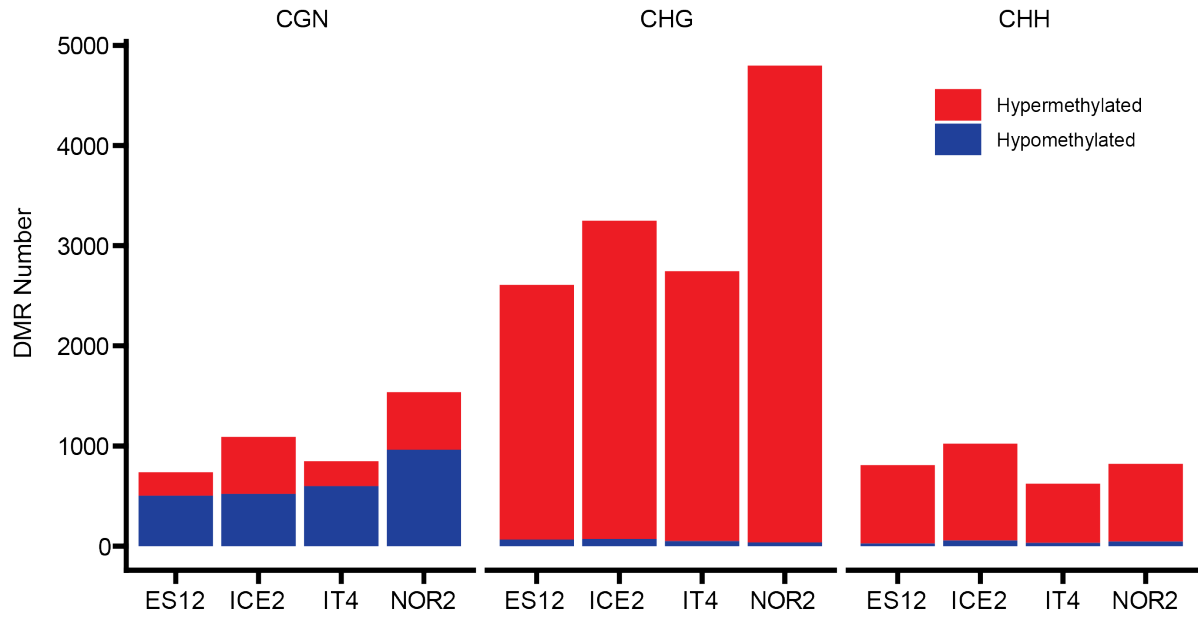

FIGURE 4. The differentially methylated regions of three methylation context in asexual reproduction of different ecotypes. X axis indicates ecotypes, ES12, ICE2, IT4, and NOR2. panels along the x axis indicate different methylation context, y axis indicates the number of DMRs. Red color indicates hypermethylation, blue color indicates hypomethylation.

We speculate that the induced hypermethylation in the CHG and CHH context could be a consequence of "self-reinforcing" chromatin interactions. In one such potential scenario, the histone methyltransferases KRYPTONITE (KYP)/(SU(VAR)3-9) HOMOLOGUE 4 (SUVH4) and its homologs SUVH5/6 could recognize the methylated CHG or CHH leading to targeted methylation of H3K9me2 (Jackson et al., 2002; Malagnac et al., 2002; Jackson et al., 2004; Johnson et al., 2007). CMT2/3 is recruited to H3K9me2 and methylates nearby CHG or CHH sites, whereas methylated CHG and CHH in turn recruit KYP and SUVH5/6 leading to reinforcement of both histone H3K9me2 and CHG/CHH DNA methylation. Furthermore, targeting of RELATIVE OF EARLY FLOWERING 6 (REF6) and putatively also other Jumonji domain-containing (Jmjc) histone demethylases, are repressed by non-CGN methylation (Miura et al., 2009; Li et al., 2016; Qiu et al., 2019), further reinforcing histone H3K9me2 (Figure 5).

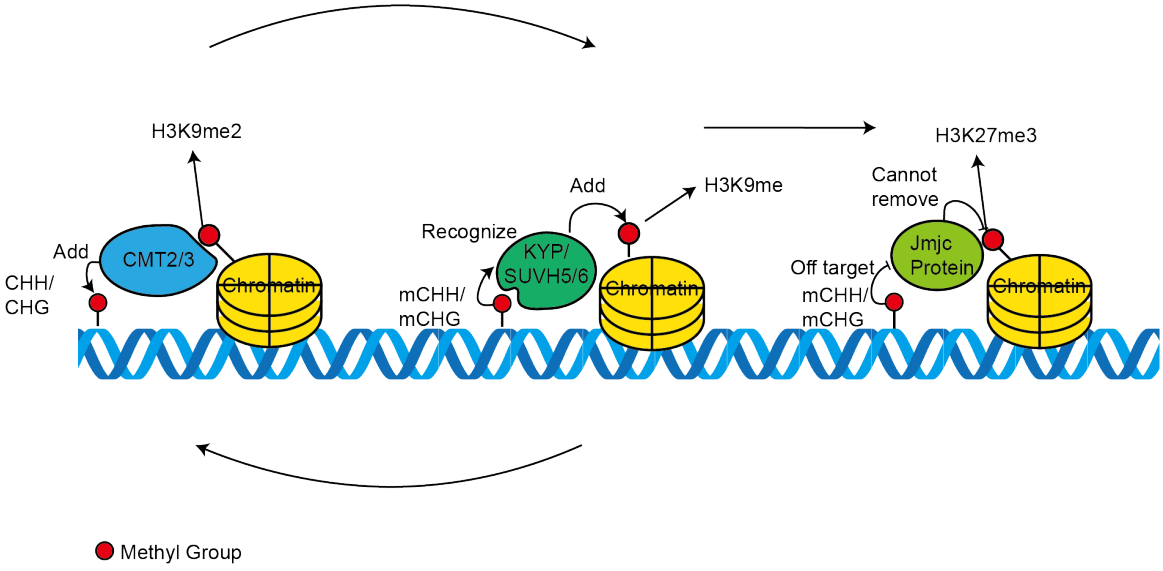

FIGURE 5. Potential mechanism that causing CHG and CHH DMRs in Asexual reproduction. H3K9 = lysine 9 on histone H3; H3K27 = lysine 27 on histone H3

The identified DMRs in the different ecotypes allowed us to call ~2500 to ~4000 treatment-induced differentially methylated genes (DMGs), most of which were associated with the CHG context (Figure 6). We noted that the identified sets of DMGs were predominantly ecotype specific. This finding was in line with the observation that the phenotypes and changes in the methylome in response to treatment were ecotype specific. The ecotype NOR2 had the highest number of DMGs, correlating with the observation that NOR2 displays the strongest induced methylation response to treatment. We tried to find a connection between induced methylation and altered transcription separately for each ecotype. We found that 10%-20% of the DEGs were DMGs, denoted DEDMGs. The DEDMGs were statistically overrepresented compared to random effect, suggesting that induced DNA methylation changes do indeed impact the expressions of at least a subset of these DEGs. GO term enrichment analysis of DEDMGs showed that translation-related categories were enriched in all 4 ecotypes. This finding suggests that temperature induced methylation changes impact translation machinery. Investigation into how the proteome is affected by these changes is of interest for further research.

Four DEDMGs occurred in all ecotypes. Interestingly, these were observed in the same DNA methylation context and at the same genomic location. The precise location suggests a targeting mechanism that upon treatment recognize these genic regions and induce DNA methylation.

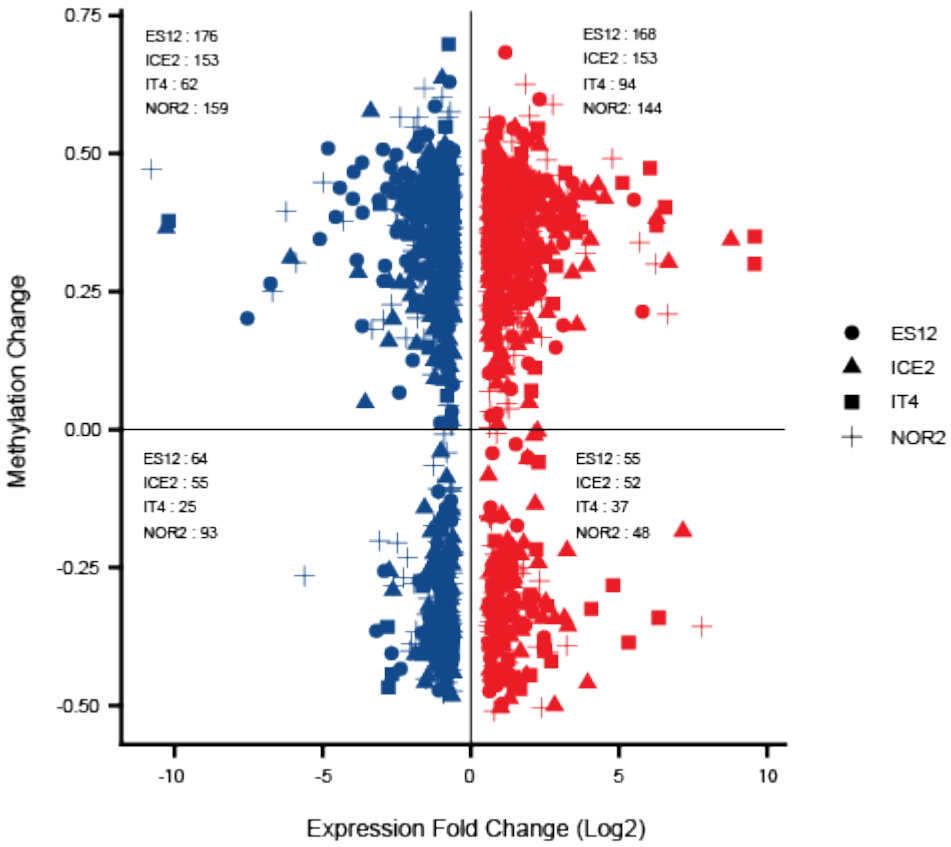

FIGURE 6. The differentially methylated and expressed genes in asexual reproduction. Different shapes indicate different ecotypes: circle: ES12, triangle ICE2, square IT4, cross: NOR2. Red indicates upregulated genes, blue indicates downregulated. Black line indicates 0 of fold change in expression or methylation. Numbers indicate genes number of different ecotypes located in quantiles.

The correlation between gene methylation status and expression has a topic of interest in previous reports. For two ecotypes of the Lotus. Li et al., identified 1500 to 2500 protein coding genes that had correlated gene expression and DNA methylation, whereof 20% were shared between the ecotypes (Li et al., 2021).

In sexual reproduction, we identified 2456 to 7403 CGN DMRs, 275 to 896 CHG DMRs, and 67 to 152 CHH DMRs, respectively (Figure 7). And DMRs tended to be slightly more hypomethylated. The identified DMRs in the different ecotypes allowed us to call ~2100 to ~5900 treatment induced DMGs, most of which were associated with the CGN context. We noted that the identified sets of DMGs in the ecotypes were predominantly ecotype specific. This finding is in line with the observation that the phenotypes and methylome changes in response to treatment were ecotype specific. The ecotype ICE2 had the highest number of

DMGs, correlating with the observation that ICE2 displays the strongest induced methylation response to treatment.

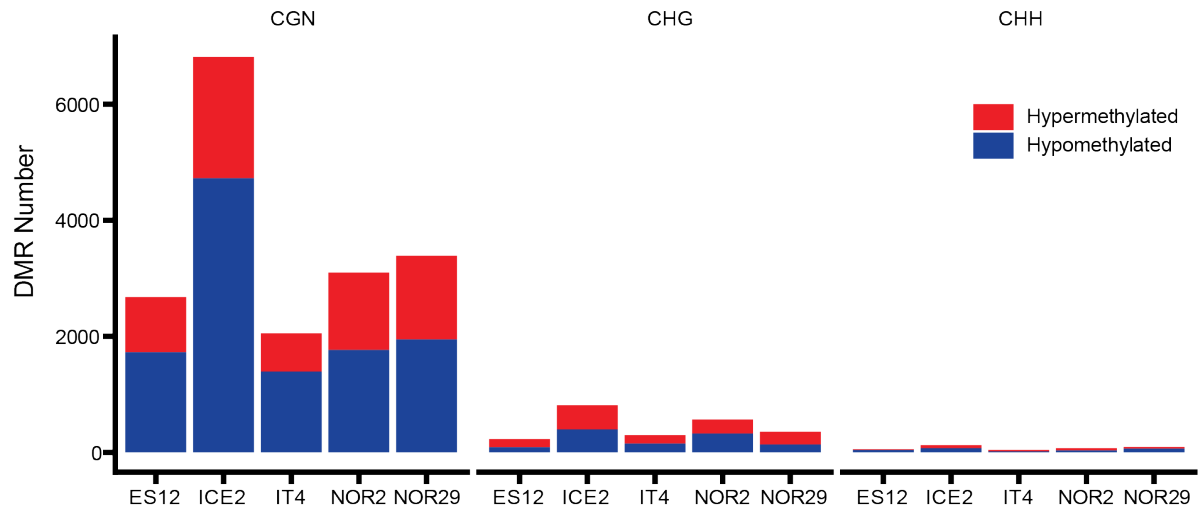

FIGURE 7. The differentially methylated regions of three methylation context in sexual reproduction of different ecotypes. X axis indicates ecotypes, ES12, ICE2, IT4, NOR2, and NOR29. panels along the x axis indicate different methylation context, y axis indicates the number of DMRs. Red color indicates hypermethylation, blue color indicates hypomethylation.

In the sexual experiment, the treatment caused transcriptome changes ranging from ~129 to ~1200 differentially expressed genes (DEGs) in the different ecotypes (Figure 8). We tried to find a connection between induced methylation and altered transcription separately for each ecotype. We found that less than 1% of these DEGs were also DMGs, denoted as DEDMGs. No DEDMGs occur in all ecotypes. The induced DEDMGs were statistically underrepresented in some ecotypes, suggesting that induced DNA methylation changes actually do not impact the expressions of at least a subset of these DEGs. Chromatin binding related GO terms were enriched in DEDMGs only in ICE2 and NOR2. This finding suggests that higher order epigenetic processes related to chromatin status are impacted by the temperature-induced methylation changes rather than specific genes. Investigation into how the chromatin status is affected by the treatment during sexual reproduction is a topic for further study.

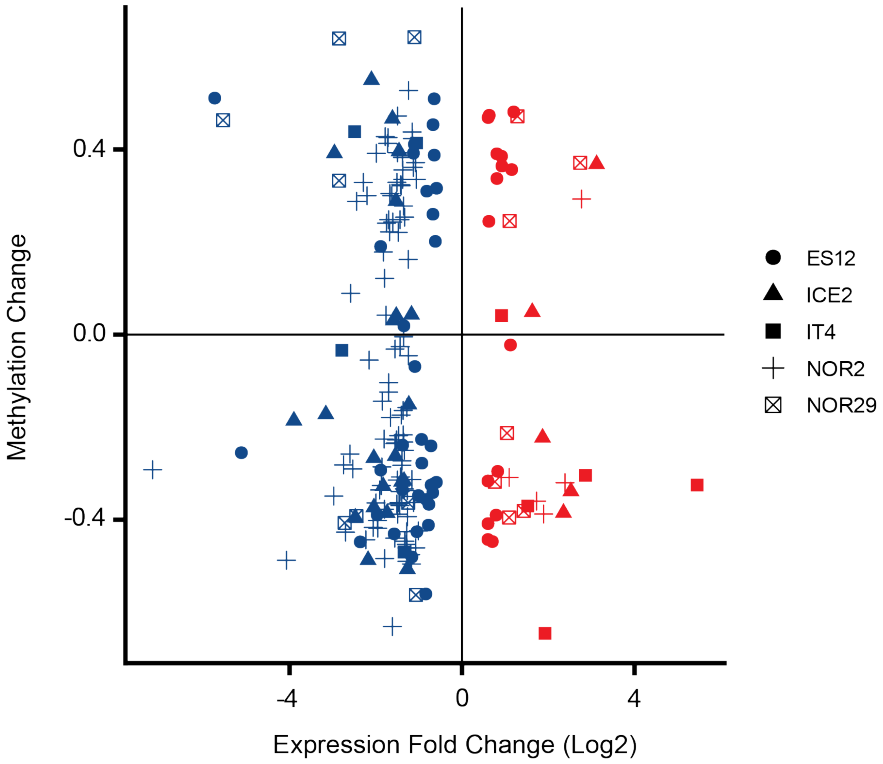

FIGURE 8. The differentially methylated and expressed genes in sexual reproduction. Different shapes indicate different ecotypes: circle: ES12, triangle ICE2, square IT4, cross: NOR2. Red indicates upregulated genes, blue indicates downregulated. Black line indicates 0 of fold change and methylation change.

A potential reason for why CGN DMRs were overwhelmingly more common than other methylation contexts is that DNA methylation mediated by METHYLTRANSFERASE 1 (MET1) is active. MET1 methylates CGN sites and maintains the methylation of CGN sites during DNA replication in plants (Kankel et al., 2003; He et al., 2011). However, MET1 lacks the function of distinguishing hemi-methylated and unmethylated CGN sites (Song et al., 2011; Du et al., 2015). Active erasing DNA methylation could be done through DNA glycosylase in base excision repair (BER) pathway. Plants have DNA glycosylases that could cut 5-mC from all the methylation context, including REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE PROTEIN 2 (DML2) and DML3 (Gong et al., 2002; Gehring et al., 2006; Ortega-Galisteo et al., 2008; Agius et al., 2006; Morales-Ruiz et al., 2006b; Penterman et al., 2007). For example, ROS1 can be recruited by the complex called INCREASED DNA METHYLATION (IDM) and H3K18Ac around the methylated cytosine (Qian et al., 2012). ROS1 can cut the methylated base and create a gap in the phosphate backbone. An unmethylated cytosine can then be inserted and ligated into a gap (Martínez-Macías et al., 2012; Lee et al., 2014; Li et al., 2015a; Andreuzza et al., 2010; Li et al., 2015b).

Genomic features in the two different reproduction modes

We confirmed that the presence of RE has a positional effect, as REs located within 2kb of a gene significantly impact the gene's expression. Previous studies generally report a reduction in expression of genes near methylated REs (Hollister and Gaut, 2009; Wang et al., 2013; Hirsch and Springer, 2017). Interestingly, we observed both up and down regulation of genes near REs that were hypermethylated in response to temperature. Thus, the impact of increased methylation of RE does not necessarily result in downregulation of a nearby gene, but can also cause upregulation.

In sexual reproduction, the positional analysis of REs also reveals that when REs is locate within 2kb of a gene, the gene's expression is influenced compared to genes without any RE.

When directly comparing the NTs of different reproductions, we found that different ecotypes actually showed different trends (Figure 9). NOR2 tend to be hypermethylated in asexual reproduction whereas ICE2 tend to have a lifted methylation level in sexual reproduction. This trend could be also verified when we calculate the methylation pattern of different genomic features (Figure 10).

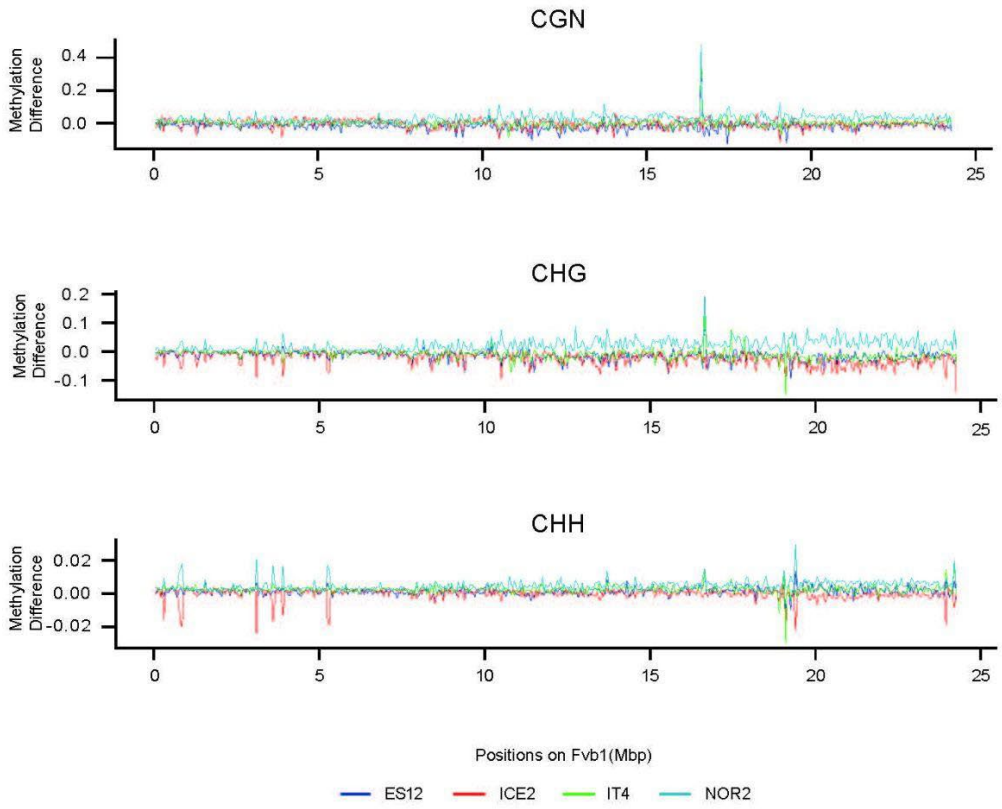

FIGURE 9. DNA methylation landscape in leaves of *Fragaria vesca* plants propagated at normal temperature conditions (18 ºC) between asexual and sexual reproduction. Methylation differences between *F. vesca* ecotypes ES12 (Spain), ICE2 (Iceland), IT4 (Italy), and NOR2 (Norway) along chromosome Fvb1. CGN, CHG and CHH methylation patterns of 50 kb windows were calculated along the chromosome using NT of AS3 as a reference.

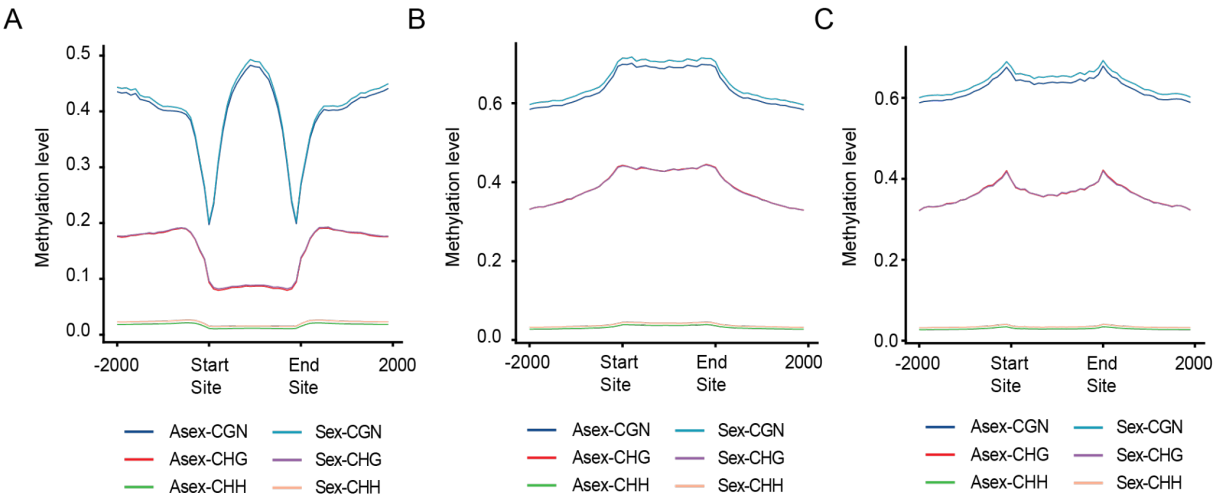

FIGURE 10. DNA methylation patterns of protein-coding genes, repetitive elements (REs), and pseudogenes in leaves of different *Fragaria vesca* ecotypes propagated at normal (18 ºC) conditions for asexual reproduction and sexual reproduction. Plots show methylation levels in protein-coding genes **(A)**, REs **(B)**, and pseudogenes **(C)** in ecotype **NOR2**. Each genomic feature, and regions 2 kb up- and downstream to these, were divided into 20 pieces to calculate methylation levels (methylated reads/total reads). Colored lines show different combinations of temperature conditions and methylation contexts (CGN, CHG, CHH).

Reprogramming of the asexual and sexual transcriptome

Epigenetics involve changes that affect gene expression and are used to describe any heritable phenotypic change in genetically identical epitypes. Such epigenetic effects can result from external factors such as changes in environmental conditions (temperature, drought and the like). When the epigenetically induced change due to environment impact on important phenological traits we call this phenomenon epigenetic memory (Carneros et al. 2017). -In this part of our study we examined the transcriptome changes due to the putative epigenetic memory of the temperature during asexual and sexual propagation in woodland strawberry (*Fragaria vesca*) ecotypes as a first step to unravel what gene products may be implicated. To this end we analyzed transcriptomes of unfolding leaves generated under two contrasting temperature conditions during stolon formation for the asexual and trough embryogenesis development to seed for the sexual propagation, as an attempt to induce an epigenetic memory of temperature. RNA-Seq and bioinformatic tools was used to unravel the genes involved in the response by (1) looking for differentially expressed genes (DEGs) using the latest known gene models, (2) finding DEGs in specific pathways and function categories such as the epigenetic machinery, and (3) finding if alternative events such as splicing variants are influenced by the treatments.

Differentially expressed genes (DEGs)

A large number of genes involved in hormone biosynthesis and signaling and terpene synthesis were affected by elevated temperatures during asexual reproduction. High temperatures are known to harm plants by causing protein misfolding, denaturing, and aggregation (Goraya et al. 2017). In our experiment we use normal to elevated temperature treatment (i.e., 18 °C *versus* 28 °C), and we saw no visible negative effects indicating damage in our experiment so this is not a heat shock-like treatment. However, the 28 °C treatment may still cause some stress to the plants compared to the 18 °C condition.

The abscisic acid (ABA)-related genes were the most enriched among hormone related genes. Abscisic acid, a plant hormone, is involved in the reaction to heat, according to extensive studies. In plants, ABA is vital for growth and stress responses. ABA could help plants adapt to their surroundings by limiting their development (Suzuki et al., 2016). ABA may improve heat tolerance by increasing antioxidant capacity and decreasing reactive oxygen species (ROS), which can cause protein denaturalization, misfolding and aggregation (Goraya et al., 2017; Lippmann et al., 2019). Other groups of genes linked to oxidoreductase activity and terpene synthesis were also overrepresented in upregulated or downregulated DEGs. Terpenes are organic chemicals that vary chemically, physically, and functionally in living organisms. Terpenes, such as gibberellin and membrane sterols, play an essential role in plants. The influence of high temperature on terpene production suggests that not only hormones, but also other complex compounds derived from terpenes may be engaged in the tolerance regulatory network (Zhou and Pichersky, 2020).

Moreover, the DEGs related to the epigenetic machinery were uniquely represented in the four ecotypes. That the ecotypes have their own distinctive sets of DEGs related to epigenetics after the temperature treatment seems to point to a fine tuning of this response to temperature. Among them, we found genes related to RdDM and DNA demethylation, histone modifiers and readers, chromatin remodeler and histone variants like XH/XS domain genes that can process double strand RNA to siRNA which is the essential part in the RdDM pathway(Finke et al., 2012), SAWADEE domain genes also can be identified as lysine readers to bind unmethylated Histone 3 lysine 4 and 9 (H3K4 and H3K9) in the RdDM pathway(Law et al., 2011, 2013), FRIGIDA-like genes, act like histone acetyltransferase in the COMPASS-like complex, can regulate the chromatin status near FLOWERING LOCUS C (FLC) region then further regulate FLC's expression(Schmitz et al., 2005; Jiang et al., 2009), plant homeodomain containing (PHD)

genes that can act as histone modification reader to recognize lysine-methylated histone H3(Sanchez and Zhou, 2011), SET domain genes involved in lysine methylation (Malagnac et al., 2002; Dillon et al., 2005), DEMETER like 1 and another DNA glycosylase involved in active DNA demethylation through base excision repair (BER)(Ikeda and Kinoshita, 2009), SWIB/MDM2 and SNF domain genes that belong to the same chromatin remodeler complex (SWI/SNF complex). Also, some of the DEGs have been reported to exert a regulatory function during temperature stress, such as the H2A variant H2A.Z. Those can be recruited by the thermosensor (PHYTOCHROME INTERACTING FACTOR 4 (PIF4);(Brickner et al., 2007; Xue et al., 2021).

Thus, the expression of genes involved in elevated temperature adaptation and epigenetic marks are affected. Importantly, the treatment caused more robust changes in the transcriptome profiles in asexual propagation compared to sexual propagation, and we note that only NOR2 ecotype had a distinct response in the sexual propagation treatment.

In addition, the examination of splicing events revealed a great number of alternative splicing events between treatments in the asexual, whereas sexual reproduction had less alternative splicing events. The most intriguing finding is that homologs of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), LATE ELONGATED HYPOCOTYL (LHY), and SHORT VEGETATIVE PHAGE (SVP) alter the utilization of respective isoforms. The A3SS event contains SOC1, while the RI event contains LHY and SVP. SOC1 has been shown to delay blooming and prolong vegetative growth in woodland strawberries via the TFL-AP1 autonomous pathway and the GA signaling pathway (Mouhu et al., 2013). Furthermore, SVP redundancy we detected indicates that the function of SVPs in woodland strawberries differs from that of the single copy in Arabidopsis thaliana. Experimental verification and functional studies of each of the *in silico* predicted homologs, as well as its spatial and temporal expression pattern, must be confirmed in future research.

Conclusions and future perspectives

We have shown that elevated temperature induced phenotype and methylome changes in four European *Fragaria vesca* ecotypes during asexual reproduction. That the phenological changes were of a lasting character and were significantly different when measured under common garden conditions indicates an epigenetic memory of the temperature during asexual reproduction. The majority of DNA methylation changes happen in the CHG and CHH contexts, and the majority of CHG and CHH are hypermethylated. Around 10%-20% of DMRs were located in gene promoter and gene-body regions. In addition, increased DNA methylation was observed both for repetitive elements and pseudogenes in response to treatment (Paper I). We also found that phenological changes can be also induced by elevated temperature during sexual reproduction (i.e., embryogenesis), but only in some ecotypes. However, the methylomes of all ecotypes were affected by the treatment. Changes in CGN methylation context were the most common DMRs and DMGs here. Over 80% of CGN DMRs were in known protein coding genes, but the number of genes having both methylation and expression change was less than 1%. DNA methylation of REs and pseudogenes was affected, but the direction of methylation change differed between ecotypes for the three methylation contexts (Paper II).

The number of differentially expressed genes (DEGs) between the two treatments may reflect differences in induced phenological change during asexual and asexual mode of reproduction (i,e epigenetic memory plasticity differences). DEGs related to the epigenetic machinery were mostly upregulated by the 28 °C treatment in the asexual experiment and downregulated in the sexual, indicating that the epigenetic machinery is impacted by temperature conditions during reproduction, but not in an equally manner between the two modes of reproduction. (Paper III). Treatment also led to alternative splicing when applied during asexual reproduction, but had less effect on splicing during the sexual mode of reproduction. A minority of the induced splicing changes were shared between ecotypes. Collectively, the results of this thesis provide evidence of the establishment of memory to environmental change (i.e. temperature sum difference) during both asexually and sexually reproduction in at least some ecotypes. Additionally, our results support that epigenetic modifications have a role in both phenotypic plasticity and environmental stress memory.

The difference in temperature response we observed between asexual and sexual reproduction may not only be due to the different modes of reproduction, but also to variations in the

experimental setup. The differential temperature treatment was reiterated for 3 consecutive asexual generations (stolons) in the asexual experiment, but only once for the sexual propagation (seed) experiment. If the effect of treatment is additive, this could lead to different strengths of memory. This could explain the weaker effects of temperature on the phenotype, methylome, and transcriptome observed in sexual reproduction experiment than in the asexual reproduction experiment. It is essential to repeat sexual reproduction for two additional generations to determine if temperature memory is additive. Another difference between the experiments is asexual reproduction occurs through vegetative propagation, while sexual reproduction occurs through embryogenesis. It is possible that epigenetic marks are reset during embryogenesis, but not vegetative propagation. Thus, the epigenetic machinery involved may differ as well as the strength of the signal needed to change phenology and epigenetic marks.

We used bisulfite sequencing and transcriptome sequencing to record methylome and transcriptome under the different treatments. We tried to correlate genomic features with DNA methylation. However, not all DNA methylation changes may be related to those genomics features we have investigated here. This suggests that DNA methylation may have more connection with other unknown/known features we have yet to examine, e.g., histone/histone modification, chromatin status, even chromosome architecture, features that are related to 3D structures of the genome and the location of the change. So we might find more functional connections to DNA methylation if we combined our data with chromatin accessibility data (ATAC-sequencing), chromatin modification data (ChIP-sequencing, or DAP-sequencing), or distant regulatory location data (enhancer, silencer, repressor, activator) in the future.

We used the latest public *F. vesca* reference genome and gene model to build our reference sequence for bisulfite mapping and DMRs annotation (Edger et al., 2018; Li et al., 2019). The reference genome and gene model are built based on the one cultivar “Hawaii 4”. The genomes of the ecotypes we used and reference genome likely differ, no matter how similar the phenotypes may be. One of the likely genome differences is in single nucleotide polymorphisms (SNPs). There have been several reports that SNP and DNA methylation have a very close correlation ((Herman and Sultan, 2016; Berbel-Filho et al., 2019). SNP and DMRs exist with certain covariation in *F. vesca* (De Kort et al., 2022). Their results suggest that methylome is the result of polygenic regulation and adaptation. So, we might find more explanations if we try to associate methylation data with SNP data of ecotypes to discover if our temperature induced DMRs could be enriched in SNP-riched regions. Alternatively, acquiring a quality genome for each ecotype would provide the best reference for calling DMRs and DMGs.

Furthermore, we only sequenced one time point from one single tissue type (young leaves). Leaf is the most abundant tissue type among plant tissues and it is very easy to sample. Many signals are produced in leaves and transferred to other tissues. For example, *FLOWERING LOCUS T* (*FT*) gene products are biosynthesized in leaf vascular tissue (phloem) and transported long distances to the shoot apex to induce flowering (Corbesier et al., 2007). Thus, only sequencing one time point for one tissue type is not sufficient to discover all the changes and mechanisms we are looking for that do not function locally in leaves and/or in a constitutive manner. Studying the meristem would likely be of importance for induction and maintenance of the epigenetic memory. Constructing pan-genome/transcriptome for multi- time points and multi- tissues could help to unveil distant regulation and temporal regulation effects of DNA methylation in the future.

In addition, we only used mRNA-sequencing which removes the ribosome RNA and most non-coding RNAs from the use of oligo-dT to enrich mRNA. With this kind of sequencing library construction strategy it is only possible to detect the non-coding/antisense transcripts originating from the intragenic region and alternative splicing events; it would exclude small RNA (~20nt) and non-coding RNA (>20nt) origin from intergenic. Both these kinds of RNA are known to play important roles in epigenetic machinery and gene expression regulation. Small RNA can be involved in RNA dependent DNA methylation (RdDM) (Erdmann and Picard, 2020). Non-coding RNA can be derived from and regulate gene expression, for example, the non-coding transcripts derived from coding region of FLOWERING LOCUS C (FLC), COOLAIR, COLDAIR, COLDWRAP (Csorba et al., 2014; Heo and Sung, 2011; Kim and Sung, 2017; Kim et al., 2017; Jeon et al., 2021). Hence, a whole transcriptome profiling including non-coding RNA sequencing such as small and long ncRNA sequencing could enable us to identify more transcripts and RNA components involved in the epigenetic machinery behind the epigenetic memory to temperature and other environmental impacts on plants.

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