Genetic and epigenetic mechanisms underlying the regulation of flowering time

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Cover image: Young *Ambrosia artemisiifolia* plant (Lejon Kralemann)

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Genetic and epigenetic mechanisms underlying the regulation of flowering time.

Abstract

Developmental transitions and responses to the environment have a tight epigenetic control. Especially the switch to flowering is important for plants because it allows (sexual) reproduction, and should not occur unless the conditions are right. External and internal signals are relayed via *FT/TFL1* genes; genes with a great impact on flowering time. The PRC1/PRC2 system plays an important role in the repression of flowering, indicated by the aberrant flowering phenotype of its mutants. Via deposition of histone modifications H2Aub1 and H3K27me3 it keeps genes stably silenced, so that transitions and responses do not happen as a result of random fluctuations in the internal or external environment.

In the first manuscript we focussed on PRC2-component MSI1 in Arabidopsis. MSI1 is the nucleosome-binding core component of PRC2 and other chromatin-modifying complexes. We found that MSI1 is also a core part of a histone de-acetylase complex together with histone de-acetylase HDA19. We further found that this complex represses the ABA-mediated salt stress response by de-acetylating the ABA receptors.

The second manuscript focused on flowering time in the invasive species *Ambrosia artemisiifolia*. During the last couple of centuries it invaded Europe, where it currently thrives mostly in the south-east. Earlier flowering populations have since been found in the north, suggesting local adaptation. We studied this early-flowering trait and found that it is inherited dominantly, and that it is maladaptive under long vegetation periods. We also identified the *FT/TFL1* genes in this species, and found that a combination of expression changes in the *FT*-like floral activator and *TFL1*-like floral repressor likely underlies the altered flowering time.

In the third manuscript we tried to shed light on repressive H2A de-ubiquitination in Arabidopsis. Previously, it has been observed that loss of UBP12/13-mediated H2A de-ubiquitination causes loss of H3K27me3 and re-activation of some PRC2 targets. We show now that this holds true on a genome-wide level, and that the genes targeted by UBP12/13 are those affected in H3K27me3 maintenance and expression. We also showed that H2Aub1 not only recruits PRC2, but likely also recruits H3K27 demethylase REF6. We show that H2Aub1 therefore puts genes in a state responsive to stimuli, and that stable repression requires its removal.

Keywords: Arabidopsis, Ambrosia, FT, TFL1, epigenetics, histone modifications, PRC1, PRC2, UBP12, UBP13

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Genetische en epigenetische mechanismen die het bloeitijdstip bepalen

Abstract

Ontwikkelingstransities en responsen op de omgeving worden sterk in toom gehouden door epigenetische mechanismen. Met name de omschakeling naar het bloeien is belangrijk voor planten omdat het de voortplanting mogelijk maakt; deze omschakeling mag niet gebeuren tenzij de condities juist zijn. Interne en externe signalen worden doorgestuurd via *FT/TFL1* genen; genen met een grote invloed op de bloeitijd. Het PRC1/2 systeem speelt een grote rol in de remming van (bloei-) genen, via de depositie van histon modificaties H2Aub1 en H3K27me3, zodat transities en reacties niet gebeuren als resultaat van willekeurige fluctuaties in de interne en externe omgeving.

In het eerste manuscript hebben we ons gericht op PRC2-onderdeel MSI1 in Arabidopsis. MSI1 is het nucleosoombindende kerneiwit van PRC2 en andere chromatinebindende complexen. Wij ontdekten dat MSI1 ook een belangrijk onderdeel van een histon de-acetylatiecomplex is, samen met histon de-acetylase HDA19. We ontdekten verder dat dit complex de ABA-gemediëerde zoutstressrespons remt door de ABA-receptoren te de-acetyleren.

Het tweede manuscript richtte zich op de bloeitijd in de invasieve soort *Ambrosia artemisiifolia*. In de laatste paar eeuwen heeft het zich gevestigd in Europa, voornamelijk in het zuidoosten. Vroeg bloeiende populaties zijn sindsdien gevonden in het noorden, wijzend op locale adaptatie. Wij bestudeerde deze vroege bloeiwijze en ontdekten dat het dominant overgeërfd wordt, en dat het niet adaptief is met lange vegetatieperiodes. Wij identificeerden ook de *FT/TFL1* genen in deze soort, en ontdekten dat een combinatie van expressiewijzigingen in de *FT*-achtige bloeiactivator en *TFL1*-achtige bloeirepressor waarschijnlijk de gewijzigde bloeitijd veroorzaakten.

In het derde manuscript wierpen wij licht op de repressieve H2A de-ubiquitinatie in Arabidopsis. Eerder had men gevonden dat op enkele genen een tekort aan UBP12/13 gemediëerde de-ubiquitinatie een vermindering van H3K27me3 en gen re-activatie veroorzaakt. Wij laten hier zien dat dit ook gebeurd op een genoom-wijd niveau. Wij ontdekten dat dit komt doordat H2Aub1 niet alleen PRC2 rekruteerd, maar ook REF6. Wij stellen een model voor waarin depositie van H2Aub1 leidt tot een responsieve staat, en dat voor stabiele remming dit verwijderd dient te worden.

Sleutelwoorden: Arabidopsis, Ambrosia, FT, TFL1, epigenetica, histon modificaties, PRC1, PRC2, UBP12, UBP13

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Dedication

To the universe, without you I would not be where I am today.

We live at a very special time . . . the only time when we can observationally verify that we live at a very special time!

Lawrence Krauss

They are in you and me; they created us, body and mind; and their preservation is the ultimate rationale for our existence. They have come a long way, those replicators. Now they go by the name of genes, and we are their survival machines.

Richard Dawkins

Contents

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I Mehdi, S; Derkacheva, M; Ramström, M; Kralemann, L; Bergquist, J; Hennig, L* (2016). The WD40 Domain Protein MSI1 Functions in a Histone Deacetylase Complex to Fine-Tune Abscisic Acid Signaling. *The Plant Cell*, vol 28, pp. 42–54.

II Kralemann, L⸸; Scalone, R⸸; Andersson, L; Hennig, L* (2018). North European invasion by common ragweed is associated with early flowering and dominant changes in *FT/TFL1* expression. *Journal of Experimental Botany*, vol 69 (10), pp. 2647-2658.

III Kralemann, L; Liu, S; Trejo-Arellano, M S; Muñoz Viana, R; Köhler, C*; Hennig, L. Removal of H2Aub1 by UBIQUITIN SPECIFIC PROTEASES 12 and 13 is required for stable Polycomb-mediated gene repression in Arabidopsis (submitted).

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⸸ Equal contribution.

The contribution of Lejon Kralemann to the papers included in this thesis was as follows:

I Minor roles in performing experiments and discussion.

II Major roles in performing experiments, discussion, writing. Performed all of the data analyses.

III Major roles in performing experiments, data analyses, discussion, and writing.

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1 Introduction

1.1 Flowering time

Flowering plants begin their life with a vegetative phase, characterized by the production of organs (leaves, roots) that are only indirectly involved in reproduction by allowing the accumulation of resources. In the reproductive phase much of the accumulated resources are channelled to the offspring. The later plants flower, the more time they have to accumulate resources, and increasing the number or size of offspring per parental plant. However, delaying flowering bears risks, since there are ubiquitous threats like herbivory, storms, flooding, drought, and especially frost-bearing winters. Early flowering bears lower risk, but lowers resources for the offspring. And it's not just a matter of creating a balance between the risk-avoidance and resource-maximizing strategies. Biotic factors can promote flowering, for instance, many angiosperms rely on animal pollinators (Ollerton *et al.*, 2011), and so flowering should be synchronized with their availability (Elzinga *et al.*, 2007). Thus, regulation of the time until the onset of flowering (i.e. flowering time) is of fundamental importance for plant reproduction.

1.1.1 The PEBP gene family

Central to the regulation of flowering time are proteins from the phosphatidylethanolamine-binding protein (PEBP) gene family, members of which have been found in all kingdoms of life (Karlgren *et al.*, 2011; Palmieri *et al.*, 2008; Serre *et al.*, 2001; Banfield *et al.*, 1998; Schoentgen & Jollès, 1995). So far, the function of the ancestral gene in the last universal common ancestor (LUCA) is unknown; however, considering the roles of PEBPS in LUCAs

descendants it seems reasonable to assume that it had a role in transducing environmental signals and regulating mitotic activity accordingly. Binding of phospholipids and phosphorylated proteins as predicted by their crystal structure and confirmed by chemical studies indicates a role in (membrane-bound) signal transduction (Nakamura *et al.*, 2014; Banfield & Brady, 2000; Banfield *et al.*, 1998; Bernier *et al.*, 1986). PEBPs may also bind and inhibit certain proteases, which has been observed for PEBPs in archaea (Palmieri *et al.*, 2010) and animals (Hengst *et al.*, 2001). Outside of the plant kingdom PEBPs are called RAF1 KINASE INHIBITOR PROTEINs (RKIPs), based on the role of PEBP1/RKIP-1 in the inhibition of the MAPK/Raf-MEK-ERK pathway (Yeung *et al.*, 1999), though the GRK2 (Lorenz *et al.*, 2003) and NFκB (Yeung *et al.*, 2001) signal transduction pathways are inhibited as well. In addition, RKIP is a positive regulator of GSK3β (Al-Mulla *et al.*, 2011). In mammals, PEBPs appear to have roles in sperm (Moffit *et al.*, 2007; Frayne *et al.*, 1998) and neuron development (Ojika *et al.*, 2000), the latter partly through cleavage of the full protein to yield the 11-amino acid HIPPOCAMPAL CHOLINERGIC NEUROSTIMULATING PEPTIDE (HCNP) (Otsuka & Ojika, 1996). In nematodes, PEBPs are involved in protection against host immune response (Morgan *et al.*, 2006), and in flies in protection against bacterial infection (Reumer *et al.*, 2009). In plants, PEBPs integrate internal and external signals to regulate developmental transitions and plant architecture (Blumel *et al.*, 2015). Early plants started with a PEBP gene now called *MOTHER OF FLOWERING LOCUS T AND TERMINAL FLOWER 1* (*MFT*). The *MFT* clade in early plant lineages has not been studied thoroughly, but there is evidence that they have a role in the regulation of germination of clonal propagules (gemmae) in the bryophyte *Marchantia polymorpha* (Eklund *et al.*, 2018), a role in the development of gamete-producing structures in the bryophyte *Physcomitrella patens* (Hedman *et al.*, 2009), and the initiation of spore-producing structures (sori) in lycophytes (Hou & Yang, 2016). By the time the last common ancestor to seed plants appeared, *MFT* had likely evolved a function in the regulation of seed germination (Nakamura *et al.*, 2015; Li *et al.*, 2014; Karlgren *et al.*, 2011; Xi & Yu, 2010). Around the same time, a duplication of *MFT* had produced the *FT/TFL1* clade (Liu *et al.*, 2016). This clade was likely tasked with the regulation of reproductive tissues, because FT/TFL1 are involved in the regulation of reproductive buds in gymnosperms (Liu *et al.*, 2016; Karlgren *et al.*, 2011). Before the divergence of gymnosperms and angiosperms the *FT/TFL1* clade split further into separate *FT* and *TFL1* clades (Liu *et al.*, 2016). In angiosperms most *FT/TFL1* genes kept their role as regulators for reproductive onset (flowering time), but gained other (lineage-specific) regulatory functions (e.g. tuberization time in potato (Gonzalez-Schain *et al.*, 2012; Navarro *et al.*, 2011),

bulbing time in onion (Lee *et al.*, 2013), and stomata opening in Arabidopsis (Ando *et al.*, 2013)).

1.1.2 Regulation of flowering time by FT/TFL1

The flowering inducing hormone 'florigen' that was described in 1937 (Chailakhyan, 1937) is now known to be encoded by *FT/TFL1* genes. In general, most *FT*-like genes are florigens / floral activators, and *TFL1*-like genes are anti-florigens / floral repressors (Karlgren *et al.*, 2011). Signals like ambient temperature, photoperiod, prolonged cold, and circadian clock are integrated to activate the *FT* gene at the appropriate time (Blumel *et al.*, 2015; Tsuji *et al.*, 2011; Turck *et al.*, 2008). The FT protein then moves through the phloem to the shoot apical meristem (SAM) where it activates genes that trigger the formation of floral meristems (Corbesier *et al.*, 2007; Abe *et al.*, 2005; Wigge *et al.*, 2005). The FT protein does not have DNA-binding activity, and relies on the bZIP transcription factor FLOWERING LOCUS D (FD, not to be confused with FLD) for targeting to the right loci (Taoka *et al.*, 2011; Muszynski *et al.*, 2006; Abe *et al.*, 2005). FT binds FD indirectly via a 14-3-3 protein, together forming the so-called florigen activation complex (FAC) (Taoka *et al.*, 2011). 14-3-3 proteins are conserved readers of phosphorylated serine and threonine in eukaryotes (de Boer *et al.*, 2013) and consistent with this is the finding that phosphorylation of FD is required for the formation of the FAC (Kawamoto *et al.*, 2015; Taoka *et al.*, 2011). TFL1-like proteins also form a complex with 14-3-3 and FD, but repress genes rather than activate them (Hanano & Goto, 2011; Simon *et al.*, 1996). TFL1-like proteins have two roles: they aid in the prevention of precocious flowering, and after flowering has started, they limit the activation of floral identity genes to certain regions so as not to convert the entire meristem to floral meristem (Pnueli *et al.*, 1998; Bradley *et al.*, 1997; Bradley *et al.*, 1996).

Apart from *FT*, Arabidopsis has another gene in the *FT*-clade: *TWIN SISTER OF FT* (*TSF*), and apart from *TFL1* two more genes in the *TFL1*-clade: *BROTHER OF FT AND TFL1* (*BFT*) and *ARABIDOPSIS THALIANA RELATIVE OF CENTRORADIALIS* (*ATC*). There is partial redundancy within clades (Yoo *et al.*, 2010; Yamaguchi *et al.*, 2005), with differences mostly in what signals the *FT* or *TFL1*-like genes respond to. Cytokinin can trigger flowering through *TSF*, but not through *FT* (D'aloia *et al.*, 2011). *BFT* is induced during salt stress, and *ATC* during short-day photoperiods, preventing precocious flowering under suboptimal conditions (Ryu *et al.*, 2014; Huang *et al.*, 2012; Ryu *et al.*, 2011). In other species, *FT/TFL1* also regulate flowering, but may respond differently to environmental signals. For instance, the rice *FT*

homologs *RFT* and *Hd3a* are induced under short days (Komiya *et al.*, 2008). And in poplar and apple age is the major floral inductive signal; decades may pass before flowering is initiated for the first time (Kotoda *et al.*, 2010; Mohamed *et al.*, 2010).

Currently there is no evidence for stimulus-sensing by the FT/TFL1 proteins themselves, but rather the *FT/TFL1* genes integrate a wide range of signals detected by other proteins. Important genes upstream of *FT/TFL1* are *FLOWERING LOCUS C* (*FLC*), *GIGANTEA* (*GI*), and *CONSTANS* (*CO*). The archetypical *A. thaliana* germinates before winter, and 'hibernates' in its vegetative rosette phase (i.e. it's a winter annual). Then it initiates flowering at the first signs of spring. The MADS-box gene *FLC* prevents precocious flowering by repressing floral activators *FT* and the FT-target *SOC1* (Bloomer & Dean, 2017). A prolonged exposure to cold (vernalisation) induces repressors of *FLC*, releasing its repression of floral activators (Bloomer & Dean, 2017). Another factor that changes from winter to spring is the daily photoperiod: the days get longer. When the daily photoperiod crosses a certain threshold GI and CO together activate *FT* (Mishra & Panigrahi, 2015).

These two pathways of regulating flowering time are called the vernalisation and photoperiod pathways, respectively. A more detailed description of the two pathways is provided in the next paragraphs. Other pathways exist (autonomous, gibberellin, temperature (Blumel *et al.*, 2015)), but are not discussed in this thesis.

1.1.3 The vernalisation pathway

Before vernalisation, *FT* is repressed by FRIGIDA (FRI) through the MADS-box protein FLC. *FRI* activates *FLC* by binding to the *FLC* locus as part of the transcriptional activating FRI complex (FRI-C) (Choi *et al.*, 2011; Kim *et al.*, 2006). FRI also recruits the nuclear cap-binding complex (CBC) (Geraldo *et al.*, 2009) and chromatin modifying factors that maintain a stable active state (Choi *et al.*, 2011; Jiang *et al.*, 2009). During vernalisation two mechanisms trigger silencing, both mediated by long non-coding RNAs. The early and seemingly non-essential silencing occurs through anti-sense transcripts collectively called *COOLAIR* that are produced from the 3' end of the *FLC* locus (Csorba *et al.*, 2014; Helliwell *et al.*, 2011; Swiezewski *et al.*, 2009). Silencing through these *COOLAIR* RNAs involve 3' RNA processing factors FLOWERING CONTROL LOCUS A (FCA), FLOWERING PROTEIN A (FPA), FLOWERING LOCUS Y (FY), CLEAVAGE STIMULATING FACTOR 64 and 77 (CSTF64 and 77), and alternative splicing factor PRE-MRNA PROCESSING 8 (PRP8)

(Marquardt *et al.*, 2014; Hornyik *et al.*, 2010; Liu *et al.*, 2010). These factors target only the *COOLAIR* transcripts, and by that trigger chromatin changes that affect *FLC* mRNA transcription (Marquardt *et al.*, 2014; Hornyik *et al.*, 2010; Liu *et al.*, 2010). A delayed but essential silencing is achieved by a sense transcript called *COLDAIR*. During vernalisation *COLDAIR* expression is induced and has a trans-acting repressive effect on the *FLC* locus (Kim *et al.*, 2017; Sheldon *et al.*, 2002). This repressive effect requires the action of a vernalisation-related variety of the chromatin-modifying Polycomb repressive complex 2 (PRC2), which contains the cold-induced PHD-finger protein VERNALIZATION INSENSITIVE 3 (VIN3) (De Lucia *et al.*, 2008; Wood *et al.*, 2006; Sung & Amasino, 2004).

Silencing of FLC is a binary, cell-autonomous process and is a low-probability event (Berry *et al.*, 2015; Angel *et al.*, 2011). Individual cells may switch their *FLC* loci from active to repressed early during the vernalisation, but in the majority of the cells *FLC* will remain active. As the period of cold persists, *FLC* will switch to the silenced state in an increasing number of cells, eventually passing a threshold that releases *FT* from repression (Berry *et al.*, 2015; Angel *et al.*, 2011).

Vernalisation evolved independently in several lineages of angiosperms, but the underlying molecular mechanisms are not well conserved (Ream *et al.*, 2012). *FLC* belongs to an angiosperm-specific MADS-box clade, and while *FLC* is involved in vernalisation in cereals and Amaranthaceae (sugar beet), it does not appear to play a major role there (Sharma *et al.*, 2017; Vogt *et al.*, 2014; Ruelens *et al.*, 2013). In Arabidopsis most of the variation in flowering time is contributed by the *FRI* locus (Johanson *et al.*, 2000). Arabidopsis summer annual varieties that pass through the winter as seeds (e.g. the common accession Columbia-0), often have a null mutation in *FRI* (Johanson *et al.*, 2000).

1.1.4 The photoperiod pathway

Similar to how various environmental and internal signals converge on *FT/TFL1* genes to trigger flowering, various signals converge on *GI* to trigger a variety of responses, including flowering via *FT/TFL1* (Mishra & Panigrahi, 2015). In the regulation of *FT* by daylength, GI has emerged as a master regulator that regulates *FT* in a *CO*-independent and *CO*-dependent manner (Mishra & Panigrahi, 2015). The expression of GI is controlled by the circadian clock, creating an mRNA peak in the late afternoon (Mizoguchi *et al.*, 2005). Stabilization of the GI protein by the blue light sensor ZEITLUPE (ZTL) depends on light (Kim *et al.*, 2007), causing the GI protein to accumulate to

higher levels under long photoperiods than under short (Sawa *et al.*, 2007). The GI protein is present at the *FT* promoter and interacts with transcriptional repressors stationed there: SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO 1 (TEM1) and TEM2, interfering with their repressive function (Sawa & Kay, 2011). Because there is more GI present under long-day photoperiods than under short, *FT* will be more strongly relieved from repression under long-day photoperiods, potentially allowing flowering.

The main activation of *FT*, however, happens in a CO-dependent manner. *CO* is repressed by CYCLING DOF FACTOR proteins (CDFs) (Imaizumi *et al.*, 2005) and especially under long photoperiods GI opposes this repression together with the light sensitive protein FLAVIN BINDING, KELCH REPEAT, F-BOX 1 (FKF1) (Fornara *et al.*, 2009; Sawa *et al.*, 2007). FKF1 and its close homologs ZTL and LOV KELCH PROTEIN 2 (LKP2) mark the CDFs for proteasomal degradation by poly-ubiquitination (Fornara *et al.*, 2009; Imaizumi *et al.*, 2005). Like GI, FKF1 is also controlled by the circadian clock and stabilized in the light, resulting in a similar but narrower protein expression pattern (Fornara *et al.*, 2009; Sawa *et al.*, 2007). The stabilization of FKF1 and its homologs is mediated by the light sensitive LOV domain that GI can bind in the light (Fornara *et al.*, 2009). This allows *CO* mRNA to accumulate more under long photoperiods than under short, but it does not fully explain the CO protein levels. CO protein levels peaks around the light to dark transition under long-day photoperiods, but not under short-day photoperiods (Valverde *et al.*, 2004). This is achieved by the regulation of CO stability by the poly-ubiquitinating CONSTITUTIVE PHOTOMORPHOGENIC 1 – SUPPRESSOR OF PHYA 105 – 1 (COP1-SPA1) complex, marking CO for degradation (Liu *et al.*, 2008). Blue light photoreceptors CRYPTOCHROME1 (CRY1) and CRY2 however interact with SPA1 to prevent this poly-ubiquitination, so that CO is only eliminated by COP1 during the dark (Liu *et al.*, 2011; Zuo *et al.*, 2011). Other factors regulating CO protein level are light sensors PHYTOCHROME A (PHYA) and PHYB, stabilizing CO under far-red light, or destabilizing CO under red light, respectively (Valverde *et al.*, 2004). The three FKF1-like proteins (FKF1, ZTL, and LKP2) that regulate *CO* expression also interact with CO and may affect CO stability (Song *et al.*, 2014; Song *et al.*, 2012). Another layer of regulation involves the AP2-like TARGET OF EAT1 (TOE1) that binds to the transcriptional activation domain of the CO protein, interfering with its activating function (Zhang *et al.*, 2015). GI counteracts this inhibition by inducing the production of *miR172*, which in turn targets the TOEs by translational silencing (Zhang *et al.*, 2015; Jung *et al.*, 2007; Aukerman & Sakai, 2003), thus activating *FT* and triggering flowering.

Homologs of proteins that in Arabidopsis induce flowering under long days, induce flowering under short days in rice. Like GI, the rice protein OsGI is an activator of *CO*-like *HEADING DATE 1* (*HD1*) (Hayama *et al.*, 2003). Unlike Arabidopsis CO, HD1 is not always an activator of *FT*-like floral activators *HEADING DATE 3a* (*HD3A*) and *RICE FT 1* (*RFT1*), but its activator/repressor nature depends on the presence of light and the length of the photoperiod (Nemoto *et al.*, 2016; Tsuji *et al.*, 2011). The monocot specific EARLY HEADING DATE 1 (EDH1) is an activator of the *FT*-like genes, and it is repressed by another monocot specific protein GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7 (GHD7) (Nemoto *et al.*, 2016). The latter requires in part the function of HD1, which explains partially why HD1 is sometimes a floral repressor (when GHD7 is present), and at other times an activator (when GHD7 is not present) (Nemoto *et al.*, 2016). As in Arabidopsis, PHYs regulate flowering through genes upstream of *FT/TFL1* (Lee *et al.*, 2016; Osugi *et al.*, 2011). The similarities in the regulation of *FT/TFL1* genes by GI and CO between rice and Arabidopsis indicate that the general mechanism of flowering control by the photoperiod pathway is conserved in flowering plants, even though different lineages have evolved the usage of new proteins on top of the conserved system.

1.2 Cellular memory

Because flowering is such an important event in the life of a plant, it should not be triggered by random fluctuations in the environment, but requires to be tightly controlled. In Arabidopsis, stable expression and repression of *FLC* and *FT*, respectively, prevents floral initiation before or during the winter (Kim *et al.*, 2009). Stable expression states are also required to prevent germination of seeds still attached to the mother plant (Footitt *et al.*, 2015; Liu *et al.*, 2007), or to prevent activation of costly stress responses when not required (Alexandre *et al.*, 2009). The ability for cells to maintain expression states can be referred to as cells having a kind of "memory". In the case of *FLC*, the gene is switched off upon sensing of cold. But after the cold period, the cells "remember" that they passed through a cold period, i.e. *FLC* stays switched off, even though the original repressive trigger is no longer present (Sheldon *et al.*, 2008; Bastow *et al.*, 2004; Gendall *et al.*, 2001). Complex mechanisms exist to maintain the "memory" as long as it is required, even allowing the "memory" to be passed on to daughter cells. Important elements maintaining this "memory" are nucleobase modifications, incorporation of variants of histone proteins, and post-

translational modifications of histone proteins. These modifications and variants can occur in virtually any DNA context, and change properties of the chromatin, making the DNA more or less accessible to the transcriptional machinery. DNA tends to be more accessible in the open conformation called euchromatin, and less accessible in the compact heterochromatin.

Histones are basic proteins that stably associate with the acidic DNA molecules, allowing organized compaction into higher order structures. They assemble into nucleosomes: heterogeneous octameric complexes, each with 147 bp of DNA wrapped around. Histones originate in the common ancestor of archaea and eukaryotes (Sandman & Reeve, 2006), and within the eukaryotic domain 5 classes of histones are well conserved: H1, H2A, H2B, H3, H4. A nucleosome always contains two H3-H4 dimers and two H2A-H2B dimers, though the exact amino acid sequence of the histones as well as the number of variants within a class may vary between species (Talbert & Henikoff, 2010). Nucleosomes are present everywhere in the genome, and often have well-defined positions relative to the transcription start site (Radman-Livaja & Rando, 2010). Histone 1, called the linker histone, is not part of the nucleosome core particle but binds the DNA at the entry/exit sites of the nucleosome. As such H1 is involved in nucleosome spacing and chromatin compaction (Hergeth & Schneider, 2015). While the nucleosome in general presents a barrier to transcription (Hodges *et al.*, 2009; Bondarenko *et al.*, 2006), histone modifications (and variants) can confer both active and repressive expression states.

1.2.1 Histone post-translational modifications

Histones consist of parts that are involved in histone-histone interactions, histone-DNA interactions, and 'tails' that stick out of the nucleosome. Post-translational modifications can occur in all three of these areas, but are more common in the tail regions (Zhao & Garcia, 2015). The modifications can affect chromatin structure and dynamics directly: modifications in the histone-histone interfaces affect the stability of the nucleosome, while modifications in areas where the DNA enters affect wrapping dynamics (Bowman & Poirier, 2014). Modifications of the histone tails may contact other nucleosomes and have an effect on higher order structures (Collepardo-Guevara *et al.*, 2015; Pepenella *et al.*, 2014). Two common modifications are lysine acetylation and phosphorylation. These modifications increase the positive charge and steric size of the histones, making interactions between histones and DNA less energetically favourable (Bowman & Poirier, 2014). Nucleosomal DNA is

constantly spontaneously wrapping and unwrapping, with higher frequency at the ends than in the middle (Li *et al.*, 2005; Anderson & Widom, 2000). Acetylation and phosphorylation promote unwrapping, increasing the probability of transcription factor binding (Bowman & Poirier, 2014). They may additionally promote nucleosome sliding and disassembly, all favouring transcription (Bowman & Poirier, 2014). Another modification that has a direct effect is mono-ubiquitination, specifically at the C-terminus of H2B (H2Bub1), because it interferes with higher order packing of chromatin (Fierz *et al.*, 2011).

Many modifications on the histone tails, however, are not directly affecting nucleosome stability and motion, but are more like tags. A tag by itself does not do anything until the appropriate reader comes along and binds the tag. A common modification is the methylation of lysine residues. For example, trimethylated lysine 4 of histone 3 (H3K4me3) is associated with transcriptional activation (Fromm & Avramova, 2014), while trimethylation of lysine 27 (H3K27me3) is associated with transcriptional repression (Mozgova & Hennig, 2015). Histone modifications are read by proteins containing specific domains. For instance, bromo or tandem PHD domains recognize acetylated lysine residues (Zeng *et al.*, 2010; Lange *et al.*, 2008; Zeng & Zhou, 2002; Dhalluin *et al.*, 1999). And methylated lysine residues are recognized by WD40, TUDOR, and CHROMO domains, amongst others (Yun *et al.*, 2011). Readers interact with amino acid residues around the modification, allowing site specificity. Even so, many readers recognize multiple modifications, especially readers of acetylated lysines (Filippakopoulos & Knapp, 2012; Kaustov *et al.*, 2011; Yun *et al.*, 2011; Vermeulen *et al.*, 2010). Readers may directly affect transcription, for instance H3K4me3 and H4 acetylation are recognized by the transcription preinitiation complex (Vermeulen *et al.*, 2007; Jacobson *et al.*, 2000). Readers can also recruit chromatin remodelling complexes to displace nucleosomes (Cavellán *et al.*, 2006). Finally, readers could also lead to the deposition of secondary marks, which may be the case for mono-ubiquitinated H2A (H2Aub1) (Dorafshan *et al.*, 2017; Zhou *et al.*, 2017a), or may cause removal of marks with opposing roles (van der Vlag & Otte, 1999).

1.2.2 The Polycomb repressive complex system

The main epigenetic repressive system involves two histone tail modifications: H2Aub1 and H3K27me3, deposited by Polycomb repressive complex 1 (PRC1) and PRC2, respectively. The mono-ubiquitin mark on H2A occurs on different (but analogous) positions in different species: K121 in Arabidopsis, K118 in Drosophila, and K119 in humans. Considering that animals (unikonts) and plants

(bikonts) both possess PRC1 and PRC2, PRC1&2 must have originated before the rise of multicellularity, and must have been present in the last eukaryotic common ancestor (LECA) (Shaver *et al.*, 2010). Millions of years of evolution have given rise to variant and accessory complexes, but the core functions of the repressive complexes have been maintained. Below I discuss complex composition and subunit function, focussing on what is conserved between lineages.

PRC2

PRC2 perhaps originated as a way to silence transposons (Shaver *et al.*, 2010), and later became co-opted to regulate differentiation and phase changes in multicellular organisms (Mozgova & Hennig, 2015). While PRC2 was present in LECA, it has subsequently been lost several times in separate unicellular lineages (Shaver *et al.*, 2010). For instance, it is absent in the Opisthokont *Saccharomyces cerivisiae* (budding yeast), the Chromalveolate *Plasmodium falciparum* (malaria parasite), and the Amoebozoan *Entamoeba histolytica* (amoebiasis parasite) (Shaver *et al.*, 2010). One component of PRC2 is present in all eukaryotes, presumably because it is a core component of multiple chromatin modifying complexes (Hennig *et al.*, 2005; Martínez-Balbás *et al.*, 1998; Tyler *et al.*, 1996). It is a nucleosome-binding protein that is called MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) in Arabidopsis, NUCLEOSOME REMODELING FACTOR SUBUNIT 55 KDA (NURF55) in Drosophila, and RETINOBLASTOMA BINDING PROTEIN 4 and 7 (RBBP4 & RBBP7) in humans (Hennig *et al.*, 2005). The enzymatic function of PRC2 is housed in the SET domain containing proteins SWINGER (SWN), CURLY LEAF (CLF), and MEDEA (MEA) in Arabidopsis, ENHANCER OF ZESTE (E(Z)) in Drosophila, and ENHANCER OF ZESTE HOMOLOG 1 and 2 (EZH1 and EZH2) in humans (Chanvivattana *et al.*, 2004; Czermin *et al.*, 2002; Kuzmichev *et al.*, 2002; Grossniklaus *et al.*, 1998; Goodrich *et al.*, 1997). A third component of PRC2 is the WD40 domain containing protein called FERTILISATION INDEPENDENT ENDOSPERM (FIE) in Arabidopsis, EXTRA SEX COMBS and EXTRA SEX COMBS-LIKE (ESC and ESCL) in Drosophila, and EMBRYONIC ECTODERM DEVELOPMENT (EED) in humans (Wang *et al.*, 2006; Ohad *et al.*, 1999; Faust *et al.*, 1998; Gutjahr *et al.*, 1995). Animal and yeast EED can recognize H3K27me3, which triggers a conformational change that activates the methyltransferase (Justin *et al.*, 2016; Jiao & Liu, 2015; Margueron *et al.*, 2009; Hansen *et al.*, 2008), aiding in the spreading and maintenance of H3K27me3. The fourth core component of PRC2

is represented by EMBRYONIC FLOWER 2 (EMF2), VERNALISATION 2 (VRN2), FERTILISATION INDEPENDENT SEED 2 (FIS2) in Arabidopsis (Gendall *et al.*, 2001; Yoshida *et al.*, 2001; Luo *et al.*, 1999), SUPPRESSOR OF ZESTE 12 (SU(Z)12) in Drosophila (Birve *et al.*, 2001), and SUZ12 in humans (Pasini *et al.*, 2004). SUZ12 is essential for PRC2 function by bridging other core proteins (Chen *et al.*, 2018b; Jiao & Liu, 2015; Nekrasov *et al.*, 2005). Additionally, mammal and plant SUZ12 homologs are involved in the recognition of active histone marks to inhibit PRC2 activity (Chen *et al.*, 2018b; Schmitges *et al.*, 2011). Finally, PRC2 may contain less conserved components, for instance the animal-specific ADIPOCYTE ENHANCER BINDING PROTEIN 2 (AEBP2), also known as JING in Drosophila (Grijzenhout *et al.*, 2016; Liu & Montell, 2001).

While there are different homologous genes encoding specific PRC2 subunits, each PRC2 complex contains only one variant of each subunit. Thus, there are different variants of PRC2 complexes, likely with different functions. For example, Arabidopsis MEA and FIS2 occur exclusively in the gametophyte specific FIS2-PRC2 complex, and prevent development of the endosperm in absence of fertilization (Chanvivattana *et al.*, 2004; Guitton *et al.*, 2004; Kohler *et al.*, 2003; Kiyosue *et al.*, 1999). VRN2-PRC2 (either with SWN or CLF) maintains the silenced state of *FLC* after vernalisation (De Lucia *et al.*, 2008). Two main variants of PRC2 in animals are distinguished by the presence of JUMONJI FAMILY ARID DOMAIN CONTAINING PROTEIN 2 (JARID2) and POLYCOMB-LIKE (PCL, multiple homologs in mammals), though both seem to promote H3K27me3 deposition by increasing the chromatin residence time of PRC2 (Youmans *et al.*, 2018; Choi *et al.*, 2017; Herz *et al.*, 2012; Hunkapiller *et al.*, 2012; Li *et al.*, 2010; Walker *et al.*, 2010; Sarma *et al.*, 2008; Nekrasov *et al.*, 2007).

PRC1 and related complexes

PRC1 is less well conserved compared to PRC2: it has not been discovered in any unicellular species, and has undergone significant divergence between lineages. As such, the function of the original PRC1 is currently unknown. The enzymatic core is conserved between plants and animals: it contains proteins from two subfamilies of REALLY INTERESTING NEW GENE (RING)-domain containing proteins that together ubiquitinate histone 2A: RING1-class and B-LYMPHOMA MOLONEY MURINE LEUKEMIA VIRUS INSERTION REGION-1 (BMI1) class. The first protein (RING1-class) is called SEX COMBS EXTRA (SCE) in Drosophila, and has two homologs in Arabidopsis

and animals: RING1A and RING1B (Sanchez-Pulido *et al.*, 2008; Xu & Shen, 2008; Gorfinkiel *et al.*, 2004; Fritsch *et al.*, 2003; Schoorlemmer *et al.*, 1997). The second component (BMI1-class) has several homologs in all species: BMI1A, BMI1B, BMI1C in Arabidopsis, POSTERIOR SEX COMBS (PSC), SUPPRESSOR OF ZESTE 2 (SU(Z)2), and LETHAL (3) 73AH (L(3)73AH) in Drosophila, and POLYCOMB GROUP RING FINGER PROTEINS 1 to 6 (PCGF1 - 6) in mammals (Gao *et al.*, 2012; Lo *et al.*, 2009; Sanchez-Pulido *et al.*, 2008; Elderkin *et al.*, 2007; Cao *et al.*, 2005; Kyba & Brock, 1998; Irminger-Finger & Nöthiger, 1995). The RING1-class possesses ubiquitin ligase activity in both animals and plants, but the BMI1-class only has this activity in plants (Bratzel *et al.*, 2010; Elderkin *et al.*, 2007; Buchwald *et al.*, 2006). In animals, it has been shown that the BMI1-class proteins stimulate the ubiquitination activity of the RING1 class proteins (Elderkin *et al.*, 2007; Buchwald *et al.*, 2006; Cao *et al.*, 2005), and in invertebrates they cause chromatin compaction (Francis *et al.*, 2004; Francis *et al.*, 2001).

In animals, the enzymatic core proteins can associate with sets of different proteins forming different complexes. The canonical PRC1 complex contains an H3K27me3-binding protein that allows H3K27me3 spreading/maintenance, and connects the PRC2 output to chromatin compaction: POLYCOMB (PC) in Drosophila, and CHROMOBOX 2 (CBX2), CBX4, CBX6, CBX7, and CBX8 in humans (Santanach Buxaderas *et al.*, 2017; Cao *et al.*, 2002; Czermin *et al.*, 2002; Bárdos *et al.*, 2000; Satijn *et al.*, 1997; Pearce *et al.*, 1992). Second, it contains a homolog of the Drosophila protein POLYHOMEOTIC (PH) (Isono *et al.*, 2005; Levine *et al.*, 2002; Franke *et al.*, 1992), a protein that functions in clustering of PRC-bound loci (Wani *et al.*, 2016). And thirdly, it contains a homolog of Drosophila SEX COMBS ON MIDLEG (SCM) (Berger *et al.*, 1999; Montini *et al.*, 1999; Bornemann *et al.*, 1996) that can interact with chromatin (Wang *et al.*, 2010; Grimm *et al.*, 2009) and can act as a platform to which other proteins can bind (Lecona *et al.*, 2015; Grimm *et al.*, 2009). The variants of PRC1 in mammals are named after the PCGF protein that is present; e.g. PRC1.2 and PRC1.4 are the canonical PRC1 complexes, and contain PCGF2 and PCGF4, respectively (Gao *et al.*, 2012). There is a non-canonical PRC1 in mammals that can have any PCGF protein, but does not have the PC, PH, and SCM components. This complex is characterized by the presence of either RING/YY1-BINDING PROTEIN (RYBP) or its homolog YY1-ASSOCIATED FACTOR 2 (YAF2), and like canonical PRC1 is involved in mono-ubiquitination of H2A and chromatin compaction (Gao *et al.*, 2012). In fact, this component is a more potent mono-ubiquitinase than the canonical PRC1, similar to the alternative dRING associated factors complex (dRAF) in Drosophila (containing the two enzymatic core proteins SCE and PSC, and additionally the

F-box protein and H3K36 demethylase dKDM2) (Gao *et al.*, 2012; Lagarou *et al.*, 2008). RYBP/YAF2 interact with YIN YANG 1 (YY1) (García *et al.*, 1999), which is the mammalian homolog of Drosophila PLEIOHOMEOTIC (PHO) and PHO-LIKE (PHOL). PHO/PHOL forms a PRC1-recruiting complex together with SCM-RELATED CONTAINING FOUR MBT DOMAINS (SFMBT) (Klymenko *et al.*, 2006; Wang *et al.*, 2004), though it's unlikely that YY1 has this role too (Kahn *et al.*, 2014).

Plants possess a plant-specific EMBRYONIC FLOWER 1 (EMF1) complex (Wang *et al.*, 2014), which is necessary for H3K27me3 maintenance and spreading and consequently for stable repression of the PRC target loci (Veluchamy *et al.*, 2016; Derkacheva *et al.*, 2013; Kim *et al.*, 2012). Unlike EED, an H3K27me3-binding activity for FIE has not been reported, and plant PRC1 lack a PC homolog. However, research has uncovered three H3K27me3 readers (LIKE HETEROCHROMATIN PROTEIN1 (LHP1), SHORT LIFE (SHL), and EARLY BOLTING IN SHORT DAYS (EBS)), all of which interact mutually exclusively with EMF1 (Li *et al.*, 2018; Wang *et al.*, 2014; Turck *et al.*, 2007; Zhang *et al.*, 2007). The spreading and maintenance function is made possible by the direct interaction of EMF1c with PRC2 (Derkacheva *et al.*, 2013; Calonje *et al.*, 2008). Loss of individual H3K27me3-readers has a mild effect on H3K27me3, but loss of EMF1 is more dramatic (Li *et al.*, 2018; Veluchamy *et al.*, 2016; Kim *et al.*, 2012). In addition to an H3K27me3 reader, EMF1c contains an eraser of the active H3K4me3 mark: JUMONJI 14 (JMJ14), JMJ15, or JMJ18 (Wang *et al.*, 2014; Lu *et al.*, 2010; Yang *et al.*, 2010; Jeong *et al.*, 2009). Thus, EMF1c may further promote silencing via counteracting deposition of active marks.

Table 1. *PRC2 and PRC1 components conserved between plants and animals*

	Arabidopsis	Drosophila	Human	Function
PRC2	MSI1	NURF55	RBBP4 RBBP7	Binds nucleosomes
	SWN CLF MEA	E(Z)	EZH1 EZH2	Methylates H3K27, H1K26 (dep. on EED isoform)
	FIE	ESC ESCL	EED (4 isoforms)	Binds H3K27me3, Activates methylase
	EMF2 VRN2 FIS2	SU(Z)12	SUZ12	Protein bridging, activates methylase
PRC1	RING1A RING1B	SCE	RING1A RING1B	Deposits H2Aub1
	BMI1A BMI1B BMI1C	PSC SU(Z)2 L(3)73AH	PCGF1 PCGF2 PCGF3 PCGF4 PCGF5 PCGF6	Compacts chromatin (invertebrate only), Stimulates RING, Deposits H2Aub1 (plants only)

1.2.3 Recruitment and hierarchy

In animals, PRC2 and PRC1 are both targeted to *HOMEOBOX* (*HOX*) genes, amongst others, and are both required for the maintenance of repression of these genes (Kwong *et al.*, 2008; Cao *et al.*, 2005; Wang *et al.*, 2004; Müller *et al.*, 2002; Wang *et al.*, 2002; Akasaka *et al.*, 2001). That, together with the fact that PRC1 contains the H3K27me3 reader PC (in animals), indicated that PRC2 is recruited first, deposits H3K27me3, in turn recruiting PRC1, leading to the deposition of H2Aub1, and finally chromatin compaction. This was later shown to be not correct (Zhou *et al.*, 2017a; Kahn *et al.*, 2016). Firstly, in both mammals and plants PRC1/H2Aub1 occur at more than a thousand loci that aren't marked with PRC2/H3K27me3 (Zhou *et al.*, 2017a; Tavares *et al.*, 2012; Schoeftner *et al.*, 2006). Secondly, the non-canonical RYBP/YAF2-PRC1 in mammals and dRAF in flies do not have an H3K27me3 reader, but they do deposit H2Aub1

and cause chromatin compaction (RYBP-PRC1) and repression (dRAF) (Gao *et al.*, 2012; Lagarou *et al.*, 2008). Thirdly, both in fly and plant PRC1 mutants about two-thirds of the genes with H3K27me3 lose this mark, while in PRC2 mutants hardly any gene loses H2Aub1 (Zhou *et al.*, 2017a; Kahn *et al.*, 2016). This indicates that in general PRC1 presence and/or action is a requirement for PRC2 recruitment, while the reverse is not true (Zhou *et al.*, 2017a; Kahn *et al.*, 2016). Indeed, this hierarchy is made possible by the finding that the accessory PRC2 components JARID2 and AEBP2 can bind H2Aub1 (Kalb *et al.*, 2014), even though PRC1-independent recruitment mechanisms exist too (Kahn *et al.*, 2016). Furthermore, a time course experiment showed that H2Aub1 is deposited before H3K27me3 in the process of X-chromosome inactivation (Almeida *et al.*, 2017).

So far, we have seen that in animals PRC1 has multiple functions. It serves to recruit PRC2 initially, it enforces PRC2 recruitment via H3K27me3 recognition to spread and maintain the mark, and it causes chromatin compaction. In plants these functions appear split between PRC1 and EMF1c. Spreading and maintenance appears more dependent on EMF1c (Li *et al.*, 2018). In addition, the BMI1-class proteins of invertebrate PRC1 contain an intrinsically disordered domain (IDD) that mediates chromatin compaction (King *et al.*, 2005). Mammal and plant BMI1-class proteins lack this region, but a functionally analogous region is present in CBX2 and EMF1, respectively (Beh *et al.*, 2012; Grau *et al.*, 2011). Thus, chromatin compaction in plants is likely mediated by EMF1c instead of PRC1 (Beh *et al.*, 2012; Kim *et al.*, 2012).

PRC1 recruitment

Because PRC1 usually arrives first, it is likely that locus specificity of PRC2 is determined (at least in part) by PRC1. The great variation and low conservation in PRC1 complexes supports this idea, and indeed different variants have different modes of recruitment. First of all, canonical PRC1 is recruited by the SCM subunit. In Drosophila, SCM targets PRC1 to the so-called Polycomb response elements (PRE), DNA elements that are sufficient for PRC recruitment (Xiao *et al.*, 2017; Kassis & Brown, 2013; Wang *et al.*, 2010). There is evidence that also mammalian PRC1 can be recruited via its SCM homologs by non-coding RNA and by DNA elements (Maezawa *et al.*, 2018; Bonasio *et al.*, 2014). A second general mechanism for recruitment of canonical PRC1 in Drosophila is through the action of PhoRC. The component PHO/PHOL has been shown to possess DNA-binding activity that targets it to PREs (Brown *et al.*, 2003; Fritsch

et al., 1999; Brown *et al.*, 1998), and recruits PRC1 via the interaction between PhoRC subunit SFMBT and PRC1 subunit SCM (Kahn *et al.*, 2014; Grimm *et al.*, 2009). Mammalian variant complex PRC1.1, also called the BCOR complex, is recruited in two different ways. One way is via its subunit KDM2B, which binds unmethylated CpG islands (CG rich areas of DNA, often found at promoters in mammals) (Zhou *et al.*, 2017c; Blackledge *et al.*, 2014; Farcas *et al.*, 2012). Loss of KDM2B leads to loss of H2Aub1 and H3K27me3 and leads to embryo lethality (Blackledge *et al.*, 2014). Flies also possess a homolog of this protein in their dRAF complex, however this protein has not been reported as a PRC1 recruiter and appears non-essential (Zheng *et al.*, 2018). The other way PRC1.1 is recruited is via the DNA-binding protein B-CELL LYMPHOMA 6 (BCL6) (Beguelin *et al.*, 2016; Hatzi *et al.*, 2013), targeting PRC1.1 to a different set of genes (Beguelin *et al.*, 2016). PRC1.3 and PRC1.5 are recruited to the X-chromosome by the *Xist* RNA, via their PSCF subunits (Almeida *et al.*, 2017). Recruitment of RYBP/YAF2-PRC1 occurs by binding to H2Aub1, presumably as a reinforcing mechanism (Almeida *et al.*, 2017; Gao *et al.*, 2012; Arrigoni *et al.*, 2006). Different complexes can also be recruited by the same factor, for instance by RE1 SILENCING TRANSCRIPTION FACTOR (REST) (Arnold *et al.*, 2013; Dietrich *et al.*, 2012; Ren & Kerppola, 2011), though in this case PRC1 appears to function without PRC2 (McGann *et al.*, 2014). In contrast to the afore-mentioned plethora of recruitment pathways in animals, we know very little about PRC1 recruitment in plants. Only the VP1/ABI3-LIKE (VAL) proteins are so far candidates for PRC1 recruitment, as well as for the recruitment of EMF1c (Merini *et al.*, 2017; Qüesta *et al.*, 2016; Yang *et al.*, 2013).

PRC2 recruitment

Most studies focus on the final product of PRC2, H3K27me3, but the intermediate H3K27me2 (and to a lesser degree H3K27me1) is actually more prevalent in the genome (Lee *et al.*, 2015; Ferrari *et al.*, 2014; Park *et al.*, 2012; Peters *et al.*, 2003). The affinity of the enzymatic core of PRC2 to its substrate is higher to K27me1 than to K27me2 (McCabe *et al.*, 2012), suggesting that stable chromatin association is required to achieve the catalysis to H3K27me3. In flies, mutations in PRC1 or PRC2 accessory factor PCL cause a reduction in H3K27me3, but an increase in H3K27me2 (Kahn *et al.*, 2016; Nekrasov *et al.*, 2007). The same is observed in the case of removal of H3K27me3 reader HP1 in *Neurospora crassa* (Jamieson *et al.*, 2016). This suggests that H3K27me1 and subsequent H3K27me2 are randomly deposited in a hit-and-run mode plausibly

to prevent random, spontaneous transcription (Lee *et al.*, 2015). But in genes, where transcription is facilitated by transcriptional activators, stronger repression is required. H3K27me3 may be a stronger repressor than H3K27me2, but does require stabilization of PRC2 first. This stabilization can be mediated by PRC1, by recognizing the H2Aub1 mark with JARID2 and AEBP2 (Youmans *et al.*, 2018; Kalb *et al.*, 2014). PRC2 may also bind to PRC1 directly, albeit transiently (Kang *et al.*, 2015; Poux *et al.*, 2001). EMF1c can interact with both PRC1 and PRC2 (Li *et al.*, 2018; Bratzel *et al.*, 2010; Xu & Shen, 2008), and so could be the main factor mediating PRC1-dependent PRC2 recruitment in plants. PRC1-independent recruitment happens through transcription factors. For instance, via the zinc fingers SNAI1 (Herranz *et al.*, 2008) and ZNF518B (Maier *et al.*, 2015). In Arabidopsis, various transcription factors have been identified that recruit PRC2 to PREs containing GA repeats and teloboxes (Jing *et al.*, 2019; Chen *et al.*, 2018a; Sasnauskas *et al.*, 2018; Xiao *et al.*, 2017; Zhou *et al.*, 2017b; Molitor *et al.*, 2016; Yuan *et al.*, 2016; Hecker *et al.*, 2015; De Lucia *et al.*, 2008). Recruitment can occur via direct interaction with a PRC2 subunit, though often also indirectly via LHP1 (Zhou *et al.*, 2017b; Molitor *et al.*, 2016; Yuan *et al.*, 2016; Hecker *et al.*, 2015). Long non-coding RNAs have also been implicated in PRC2 recruitment in animals and plants (Heo & Sung, 2011; Tsai *et al.*, 2010; Rinn *et al.*, 2007), though more recent studies showed that PRC2 binds nascent RNA rather unspecifically, and that RNA binding inhibits PRC2 activity (Beltran *et al.*, 2016; Cifuentes-Rojas *et al.*, 2014; Kaneko *et al.*, 2014).

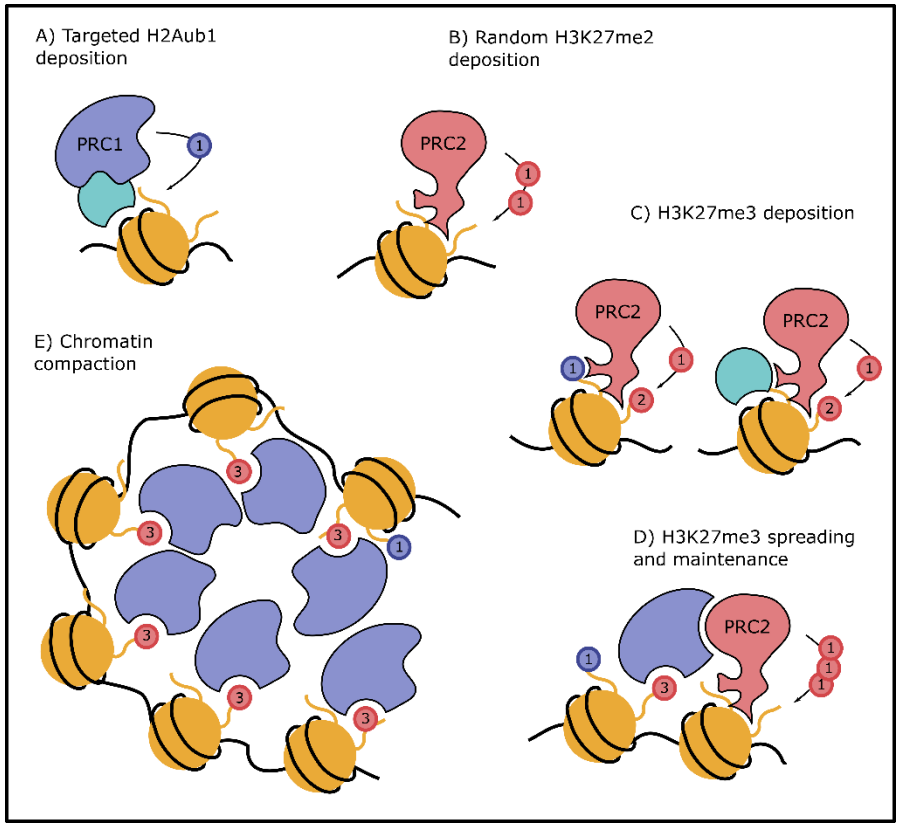

Figure 1. Conserved PRC function. A) PRC1 is targeted to specific loci by DNA (or RNA) binding non-core components like SCM (-like) in animals, or VAL1 in plants, and then deposits H2Aub1 (small blue circle). B) PRC2 has autonomous nucleosome binding activity, binding chromatin randomly and briefly, and depositing H3K27me1 and H3K27me2 (small red circles). C) Increased residence time on the chromatin allows deposition of H3K27me3. In animals, residence time is increased by the recognition of H2Aub1 by accessory subunits AEBP2 and JARID2. In plants, such a PRC2-associated H2Aub1 reader has not been identified. PRC2 can also be recruited by DNA-binding factors, allowing targeting independently of PRC1. D) H3K27me3-readers allow spreading of the H3K27me3 mark beyond the nucleation site, and to maintain it during DNA replication. The blue complex is PRC1 in animals (recognizing H3K27me3 through PC), or EMF1c in plants (through LHP1, EBS, and SHL), which interacts with PRC2 to deposit H3K27me3 on an adjacent nucleosome. In mammals the PRC2 subunit EED can recognize H3K27me3, allowing PRC2 to spread the mark without any additional complex. E) Nucleosomes of near and distant places can cluster together, compacting the chromatin. Local compaction depends on intrinsically disordered domains (IDD) present in invertebrate BMI1-class proteins, in CBX2 in mammals, and in EMF1 in plants. IDDs have the potential to form polymers, providing a mechanism for the compaction mediated by PRC1. Similarly, PH was reported to form polymers and to cluster together (distant) PRC1-bound loci.

1.2.4 Histone de-ubiquitination

Like all histone marks, H2Aub1 has its erasers. Genes kept repressed by the PRC system do not necessarily need to stay repressed for ever, but require to be reactivated in response to environmental or developmental signals. For reactivation, the action of the PRCs needs to be reversed. It has been well established that reactivation requires H3K27me3 demethylation (Van der Meulen *et al.*, 2015; He *et al.*, 2012b), and so one could imagine the same for H2Aub1 erasers. However, the relationship between H2Aub1 and expression is not straightforward. Both in animals and in plants erasers have been described that remove the H2Aub1 mark, and yet this removal is not associated with gene activation, but rather with repression and increased levels of H3K27me3 (Derkacheva *et al.*, 2016; Scheuermann *et al.*, 2010).

It is unclear whether H2Aub1 is truly a repressive mark. Loss of H2Aub1 has been implicated in the release of RNA polymerase II from its poised state in mammals, though the concomitant (but milder) loss of H3K27me3 could have caused this de-repression instead (Stock *et al.*, 2007). On the other hand, several studies in animals showed that enzymatically inactive PRC1 is still able to confer gene repression and chromatin compaction (Illingworth *et al.*, 2015; Pengelly *et al.*, 2015; Eskeland *et al.*, 2010). As mentioned before, PRC2 can be recruited by H2Aub1 via JARID2 and AEBP2 (Kalb *et al.*, 2014), though this does not mean that H2Aub1 has to remain at a locus after PRC2 recruitment. Once H3K27me3 has been established, H3K27me3 can act as a PRC2 recruiting signal via EED and PCL in animals, and via EMF1c in plants. At that point H2Aub1 could become dispensable. In Arabidopsis, loci with H3K27me3 and H2Aub1 have a higher average expression than loci without H2Aub1, indicating that H2Aub1 rather interferes with repression (Zhou *et al.*, 2017a). Together this suggests that H2Aub1 is not just required to push the PRC system in the direction of repression and chromatin compaction, but that it has another role. It is possible that H2Aub1 or an H2Aub1-binding factor is limiting, and release of H2Aub1 from a locus that is already repressed makes it available to allow repression of another locus (Scheuermann *et al.*, 2012). Alternatively, H2Aub1 interferes with the repression downstream of PRC2 recruitment, and requires to be removed after deposition for proper repression (Scheuermann *et al.*, 2012).

In flies, the repressive de-ubiquitination is mediated by the Polycomb repressive de-ubiquitinase complex (PR-DUB) (Scheuermann *et al.*, 2010). It is comprised of the de-ubiquitinase CALYPSO and the chromatin-binding ADDITIONAL SEX COMBS (ASX), that both are required for H2A de-ubiquitination and HOX gene repression (De *et al.*, 2019; Scheuermann *et al.*, 2010; Sinclair *et al.*, 1998). The human homolog of CALYPSO, BRCA1-

ASSOCIATED PROTEIN 1 (BAP1), and the ASX homologs ASXL1, ASXL2, and ASXL3 could similarly interact and de-ubiquitinate H2A (Srivastava *et al.*, 2015; Lai & Wang, 2013; Scheuermann *et al.*, 2010). In plants a similar system was found, albeit with non-homologous proteins (Derkacheva *et al.*, 2016). UBIQUITIN SPECIFIC PROTEASES 12 and 13 have been identified as interactors of LHP1, and mutation of the genes encoding these de-ubiquitinases causes loss of H3K27me3 and de-repression of some PRC2 targets (Derkacheva *et al.*, 2016). Research in mammals showed that loss of the ASXLs also causes loss of repression and H3K27me3 (Lai & Wang, 2013; Abdel-Wahab *et al.*, 2012). However, loss of BAP1 has the opposite effect (Campagne *et al.*, 2019; LaFave *et al.*, 2015). Genome-wide analyses of the consequences of loss of H2A de-ubiquitination on expression and H3K27me3 in flies and plants is required to determine whether the reported repressive H2A de-ubiquitination truly exists.

1.2.5 PRC-mediated gene repression

The role of the PRC system is to provide repression stability. In general the PRCs do not cause the initial repression, but rather ensure that genes do not get spontaneously reactivated (Helliwell *et al.*, 2011; Eskeland *et al.*, 2010; Schubert *et al.*, 2006; Gendall *et al.*, 2001). This stable repressive state is achieved through a combination of different mechanisms. Firstly, animal PRC1 interferes with the assembly of the pre-initiation complex of RNA polymerase II (Lehmann *et al.*, 2012). The PRCs also interfere with transcriptional elongation at bivalent genes (Stock *et al.*, 2007). In addition to affecting transcription directly, the PRC system counters the deposition of active histone marks. For instance, the presence of H3K27me3 prevents deposition of H3K27Ac because the two marks cannot coexist, and because PC inhibits the acetylase CBP directly (Tie *et al.*, 2016; Pasini *et al.*, 2010). And in mammals PRC2 recruits the H3K4me3 demethylase RBP2 (Pasini *et al.*, 2008), while in plants the EMF1 complex possesses H3K4me3 demethylase activity.

However, the main mechanism of repression is chromatin compaction (Lau *et al.*, 2017; Shao *et al.*, 1999). *In vitro* studies have shown that animal PRC1 can compact nucleosomal arrays, a process that does not require histone modifications (Eskeland *et al.*, 2010; Francis *et al.*, 2004). This compaction is dependent on an IDD that resides in BMI1-class proteins in invertebrates, in CBX2 in mammals, and in EMF1 in plants (Beh *et al.*, 2012; Grau *et al.*, 2011; King *et al.*, 2005). In addition to PRC1, mammalian PRC2 has also been reported to compact chromatin *in vitro* through EZH1, a process that does require histone

tails (Margueron *et al.*, 2008). EZH1 is only a weak H3K27 methylase, but a strong chromatin compactor, while the opposite is true for EZH2 (Margueron *et al.*, 2008). However, since EZH1 is often associated with the active H3K4me3 mark, and has been shown to stimulate RNA polymerase II elongation (Mousavi *et al.*, 2012), it is unclear whether EZH1 has a major role in repression. Compaction and repression might require H3K27me3 together with PRC1 or EMF1c (Kim *et al.*, 2012; Eskeland *et al.*, 2010; Schubert *et al.*, 2006), as well as recruitment of variant histone H1.2 in mammals (Kim *et al.*, 2015a). In addition, PRC2 has been shown to methylate H1K26, which is required for L3MBTL1-mediated chromatin compaction (Trojer *et al.*, 2007; Kuzmichev *et al.*, 2004). PRCs do not only cause local condensation of the chromatin, but also cause higher order reorganization of the chromatin by creating clusters of PRC-bound loci (reviewed in (Entrevan *et al.*, 2016)). A critical factor for clustering in animals is PH, though this clustering has also been observed in plants which do not have a PH homolog (Wani *et al.*, 2016; Rosa *et al.*, 2013). The mode of action of PH is through polymerisation, linking multiple chromatin-bound PRC1 complexes together (Robinson *et al.*, 2012). Recently it has been found that the mode of chromatin compaction of CBX2 is also by polymerization through its IDD (Tatavosian *et al.*, 2019), indicating that the same may be true for EMF1.

In the transition to flowering, the PRC system plays an important role as evidenced by the early flowering phenotype of PRC2 and EMF1c mutants (Wang *et al.*, 2014; Gaudin *et al.*, 2001; Yoshida *et al.*, 2001; Goodrich *et al.*, 1997), and late flowering phenotype of PRC1 mutants (Shen *et al.*, 2014). In the vegetative phase of Arabidopsis, *FT* repression is maintained by FLC through recruiting EMF1c (Wang *et al.*, 2014). PRC2 may then be recruited via EMF1 or LHP1 interactions (Derkacheva *et al.*, 2013; Calonje *et al.*, 2008). There is no direct evidence for PRC1 recruitment to *FT*. *FT* repression also requires the action of H3K4me3 demethylases (Yang *et al.*, 2010; Jeong *et al.*, 2009). During vernalisation, *FLC* becomes silenced and targeted by VAL1 recruiting a de-acetylase complex (Qüesta *et al.*, 2016). VRN2 is already present at the locus before vernalisation, but the VRN2-PRC2 complex only becomes constituted during vernalisation, resulting in H3K27me3 deposition (Heo & Sung, 2011; De Lucia *et al.*, 2008). VAL1 interacts with BMI1A/B/C, LHP1, and MSI1 (Chen *et al.*, 2018a; Yuan *et al.*, 2016; Yang *et al.*, 2013), so there are multiple ways PRC1 and PRC2 can be recruited to the locus. In addition, long non-coding RNAs have been suggested to directly (Heo & Sung, 2011), and indirectly (Tian *et al.*, 2019) recruit PRC2 at *FLC*. After PRC targeting, the two *FLC* loci on both chromosomes move together, indicating the formation of PRC clusters (Rosa *et al.*, 2013). After *FLC* is silenced, *FT* can be activated by the photoperiod

pathway. The induction of *FT* involves the removal of the H3K27me3 mark by REF6 (Lu *et al.*, 2011).

2 Aims of the study

1. The PRC2-component MSI1 functions in several chromatin modifying complexes. Previously, we found that it interacts with HISTONE DEACETYLASE 19 (HDA19) (Derkacheva *et al.*, 2013), indicating it might be part of a histone deacetylase complex too. An earlier study implicated MSI1 in regulating the ABA-response (Alexandre *et al.*, 2009). We aimed to determine whether MSI1 was part of an HDA19 complex, and whether it regulates the ABA response through this complex.

2. Recently, early flowering invasive populations of *Ambrosia artemisiifolia* have been discovered in Northern Europe (Scalone *et al.*, 2016; Leiblein-Wild & Tackenberg, 2014). We aimed to determine whether changes in *FT/TFL1* expression caused the early flowering phenotype and contributed to its spread northward.

3. Histone 2A de-ubiquitinases UBP12/13 have been shown to maintain H3K27me3 levels and the repressive state on certain PRC2 targets, just like CALYPSO in Drosophila (Derkacheva *et al.*, 2016; Alexandre *et al.*, 2009). However, the mechanism of UBP12/13 function in repression was unclear, which I aimed to unravel in this thesis.

3 Results and Discussion

3.1 PRC2-component MSI1 is part of a histone deacetylase complex (I)

Previously, our lab identified interaction partners of MSI1 by immunoprecipitation followed by mass spectrometry (IP-MS) (Derkacheva *et al.*, 2013). One of the interactors is HISTONE DEACETYLASE 19 (HDA19), homologous to REDUCED POTASSIUM DEFICIENCY 3 (RPD3) in yeast and animals. As mentioned before, MSI1 and related proteins are a core part of multiple chromatin modifying complexes and present in all eukaryotes. In addition to being part of PRC2 and chromatin assembly factor 1 complex (CAF1) (Kaya *et al.*, 2001), this interaction indicates it might also be part of an RPD3 complex, also known as a SWI-INDEPENDENT 3 histone deacetylase (SIN3-HDAC) complex. Indeed, previous studies found an interaction between HDA19 and two SIN3-like proteins (Wang *et al.*, 2013; Song *et al.*, 2005). In animals, the complex also contains SIN3-ASSOCIATED POLYPEPTIDE OF 18 KDA (SAP18) (Zhang *et al.*, 1997), and like-wise, an interaction between HDA19 and the Arabidopsis SAP18 has been found (Hill *et al.*, 2008; Song & Galbraith, 2006). The large RPD3 complex in yeast (RPD3L) contains RXT3, a protein with an early eukaryotic origin that has been lost in animals (Perrella *et al.*, 2013). Partial homology has been found in Arabidopsis protein HISTONE DEACETYLASE COMPLEX 1 (HDC1), a protein that also interacts with HDA19 (Perrella *et al.*, 2013). Here we confirmed the interaction between MSI1 and HDA19 by performing an IP-MS experiment using HDA19 as bait, as well as the interaction with all six SIN3-like proteins and HDC1 (Paper I - Table 1). Native PAGE, co-IP, and yeast-two-hybrid (Y2H) further confirmed their interaction (Paper I - Figure 1). A strong correlation between expression patterns

of MSI1 and HDA19 expression showed that they can also interact *in vivo* (Paper I - Figure S1).

MSI1, HDA19, HDC1, and SAP18 have all been implicated in ABA-mediated drought stress response (Perrella *et al.*, 2013; Chen & Wu, 2010; Alexandre *et al.*, 2009; Song & Galbraith, 2006; Song *et al.*, 2005), and so we hypothesized that they affect this response via histone de-acetylation. We tested this by analysing the response to ABA of known ABA-responsive genes in wild type, an *msi1* anti-sense (as) line, and an *hda19* mutant. We showed that the expression increased more strongly in the as-line/mutant, indicating that the MSI1-HDA19 complex attenuates the ABA response (Paper I – Figure 2). We then tested the expression level of ABA-receptor genes, and found that in the as-line/mutant this expression level was increased (Paper I – Figure 3). This indicates that the attenuation of the ABA response happens through repression of ABA-receptors. To test whether this repression was a consequence of de-acetylation of the genes encoding the ABA-receptors, we performed chromatin immunoprecipitation followed by quantitative polymerase chain reaction (ChIP-qPCR). We indeed found that MSI1 and HDA19 bound at the chromatin of the ABA-receptors (Paper I – Figure 4), and that the as-line/mutant had higher acetylation levels here (Paper I – Figure 5), showing that the HDA19 complex attenuates the ABA-response via de-acetylation of the ABA-receptors. Finally we show that the as-line/mutant plants were more tolerant to salt stress, and that general histone-de-acetylation inhibition improves salt tolerance of wild type plants, but not of *hda19* mutant plants (Paper I – Figure 6). The as-line did not lack the HDA19 complex completely, and as such was still responsive to the de-acetylation inhibitor. But the level of tolerance was similar to that of the *hda19* mutant, indicating that another MSI1-containing complex, perhaps PRC2, also plays a role in inhibiting the salt stress response.

Histone de-acetylation can contribute to the PRC1/2 repressive system, and as such can be required for the regulation of developmental transitions and stress responses (Basta & Rauchman, 2017; Barnes *et al.*, 2014; Jung *et al.*, 2010b; Zhou *et al.*, 2005; Ahringer, 2000). Histone de-acetylation is required for PRC2 function since an acetylated lysine residue cannot get methylated (Kim *et al.*, 2015b; Reynolds *et al.*, 2012; Jung *et al.*, 2010a). In turn, H3K27me3 and PC prevent histone acetylation (Tie *et al.*, 2016; Pasini *et al.*, 2010). However, this does not mean all PRC2 and histone deacetylase complexes work towards the same end. EMF2-PRC2 has a floral repressive function as it targets floral activators *FT* and *AGL19*, while VRN2-PRC2 activates flowering by repressing FLC (Jiang *et al.*, 2008; Schonrock *et al.*, 2006; Gendall *et al.*, 2001; Chandler *et al.*, 1996). HDA5 and 6 target *FLC*, and as such are floral activators (Luo *et al.*, 2015; Gu *et al.*, 2011), while the de-acetylation of *AGL19* makes HDA9 a

floral repressor (Kim *et al.*, 2013). And HDA19 is both a floral activator and repressor, depending on the photoperiod (Ning *et al.*, 2019).

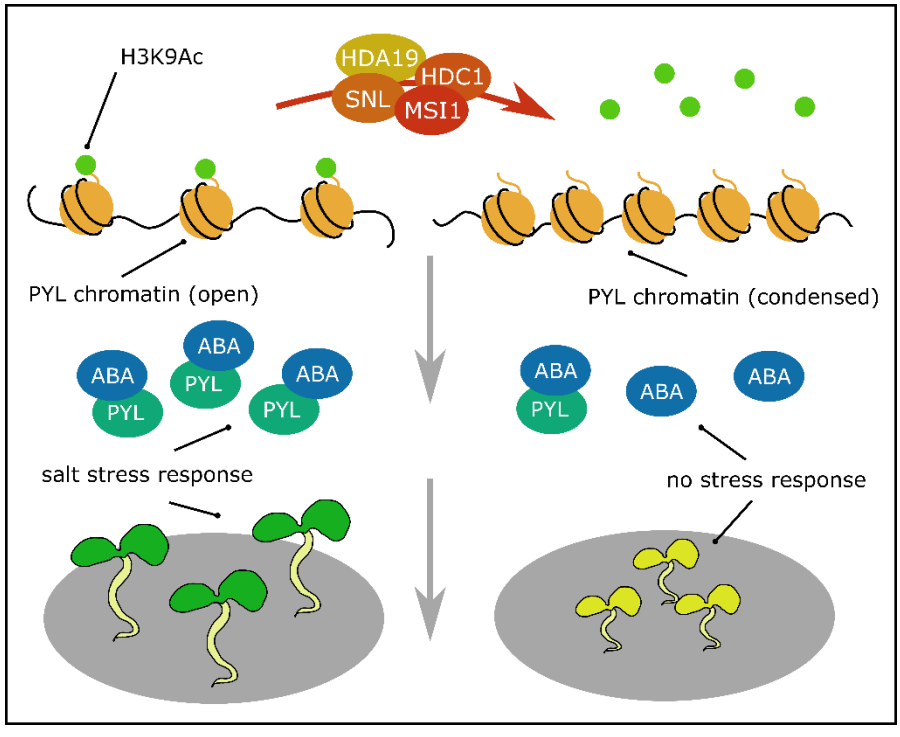

Figure 2. The HDA19-MSI1 complex inhibits the ABA mediated salt stress response. The complex containing MSI1, HDA19, HDC1, and (presumably one of six) SIN3-like proteins ('SNL' in the figure) removes acetylation from H3K9 from *PYL4*, *PYL5*, and *PYL6* ('PYL' in the figure). This leads to repression of the genes, presumably via chromatin compaction. As a result few ABA-receptors are being produced, preventing the salt stress response.

3.2 Changes in *FT/TFL1* expression are associated with invasion (II)

Plants recognize certain environmental signals to flower on time. Outside of their native habitat the same signals may not trigger timely flowering. The invasive species *Ambrosia artemisiifolia*, native to North America, has a European distribution that is mainly restricted to the south-east. It is a short day plant: it flowers when the daily photoperiod falls below a certain value in the summer. In Northern Europe the daily photoperiods in the summer are longer, which makes the plant flower later in the year than in the south. This, combined with earlier damaging cold at higher latitudes restricted the distribution of

Ambrosia to the south. However, small populations with earlier flowering time have been found recently in the North (Scalone *et al.*, 2016; Leiblein-Wild & Tackenberg, 2014).

We grew plants from (Northern) invasive and native populations under controlled conditions inductive to flowering for the native population, and found that the invasive population flowered about a month earlier (Paper II – Figure 1). By also growing offspring of crosses between invasive and native populations, we determined that the early flowering trait is dominant or over-dominant for female or male flowers, respectively. Under flowering conditions inductive for native populations, early flowering was clearly maladaptive: it resulted in a smaller final size, and a subsequent lower seed production (Paper II – Figure 2). A dominant, maladaptive allele would likely be rapidly purged, indicating that it probably originated recently. Because *FT/TFL1* genes are likely candidates through which early flowering would be achieved, we attempted to identify the homologs in this species. Using 5' and 3' RACE and sequencing we identified two *FT/TFL1* genes that were named *FTL1* and *FTL2*. A third potential *FTL* gene was highly divergent and possibly non-coding, so it was not further analysed. To determine the function of these genes we performed a phylogenetic analysis with the predicted amino-acid sequences of 172 PEBP proteins from 33 species/16 families of plants. We found that FTL1 belongs to the FT-clade, and FTL2 to the TFL1 clade (Paper II – Figure 3). While most FT-like proteins were activators and most TFL1-like protein were repressors, 12% did not follow this pattern. We therefore predicted the function of the FT/TFL1 proteins within the two clades using the amino acid sequences of FT/TFL1 proteins with known function. Our method called 92 out of 95 proteins with known function correctly, revealing that the method is reliable (Paper II – Table S3, Table S4). Using this method we confirmed our initial prediction of FTL1 and FTL2 function (Paper II – Figure S7, Table S3, Table S4). A final confirmation was made by heterologous expression of *FTL1* and *FTL2* in Arabidopsis (Paper II – Figure 4), which supported the *in silico* prediction. We next investigated whether the floral activator *FTL1* and floral repressor *FTL2* were differentially expressed in native and invasive plants, and so we performed a time-course gene expression analysis. We found both an earlier increase in the expression of the floral activator, and a decrease in the expression of the repressor (Paper II – Figure 5). We concluded that probably a recent dominant mutation changed the expression of *FT/TFL1* genes, and that this allowed the species to spread further northward.

3.3 H2A de-ubiquitination is required for stable PRC1/2-mediated repression (III)

As is the case for PRC2 mutants, *ubp12 ubp13* double mutants display early flowering and short stature (Cui *et al.*, 2013). UBP12/13 interact with LHP1, and are required for H3K27me3 maintenance and repression of some PRC2 targets, akin to the Drosophila protein Calypso. However, neither the studies on UBP12/13 nor on Calypso were genome-wide, so the possibility remained that what was observed were indirect effects of histone 2A de-ubiquitination. In fact, no mechanism was yet identified of how removal of H2Aub1, the product of PRC1, could support PRC1/2 mediated repression. Neither study addressed what happened to the H2Aub1 mark on the tested PRC2 target genes, which sparked the hypothesis that perhaps H2Aub1 is removed from other genes or genomic regions, releasing either ubiquitin or an H2Aub1-binding factor that can then bind at the tested PRC2 target genes. Alternatively it was hypothesized that H2Aub1 needs to be deposited and then later removed from the same locus, though no evidence was present to warrant hypothesizing about why the removal should be required. In our study we wanted to test these hypotheses, and if the second hypothesis was true, to test what mechanism could explain the requirement for H2Aub1 removal.

Firstly, I generated RNA-seq data of single and double *ubp12* and *ubp13* mutants. The gene deregulation reflected the phenotypes of the mutants in the sense that the single mutants were wild type-like (Paper III – Figure S1), while the double mutants had a strong abnormal phenotype. The double mutants were enriched for genes related to stimulus response (Paper III – Table S2). I furthermore found that genes upregulated in *ubp12 ubp13* mutants were in general also upregulated in PRC1, PRC2, and EMF1c mutants, further lending credence to the hypothesis that they work together to repress genes (Paper III – Figure 1). I then wanted to determine which loci were targeted by UBP12/13, and generated H2Aub1 and H3K27me3 ChIP-seq data. I found that most UBP12/13 targets contain H3K27me3, and that in the mutant these genes tend to be upregulated (Paper III – Figure 1).

As previously discussed, H2Aub1 may not be a repressive mark. And certainly, it being a repressive mark would make it more difficult to envision a model where removal of H2Aub1 would have a repressive effect on the same locus. I therefore tested the hypothesis that H2Aub1 is not a repressive mark by using previously published ChIP-seq and RNA-seq data. I found that genes with H2Aub1 tended to have a higher expression than genes without (Paper III – Figure 2). I also discovered that genes that were upregulated in PRC1 mutants were those marked with PRC1-dependent H3K27me3, not those with only

H2Aub1 (Paper III – Figure 2). I thus concluded that PRC1/H2Aub1 causes repression via recruiting PRC2, but beyond that H2Aub1 does not have a repressive effect. Furthermore, I addressed the question why some loci require PRC1 to recruit PRC2, while other loci do not. I analysed previously published data, and found that both PRC1-dependent and independent genes were equally repressed, but that in the absence of PRC2 only the PRC1-dependent genes were upregulated (Paper III – Figure 2). This indicated that PRC1-independent genes are pre-repressed, in contrast to PRC1-dependent genes.

This data revealing that H2Aub1 is not a repressive mark *per se*, is consistent with the idea that H2A de-ubiquitination is required for repression. To unravel the mechanism explaining the requirement of H2Aub1 removal for repression, I investigated the distribution of the H3K27me3 mark at UBP12/13 targets in *ubp12/13* mutants. I found that in the mutants the level of the mark is decreased, agreeing with the fact that UBP12/13 targets are upregulated in the mutant (Paper III – Figure 3). To test a possible role of UBP12/13 in H3K27me3 spreading, I tested whether H3K27me3 was preferably lost from the 3' end. Our results showed that this was not the case, in fact the loss was greater at the 5' end, but only mildly so (Paper III – Figure 3). I then tested whether de-ubiquitination could prevent active removal of H3K27me3 by a demethylase. I first tested whether genes with H2Aub1 were enriched for the K27 demethylase REF6, and indeed this was the case (Paper III – Figure 3). And consequently, genes with H2Aub1 tended to be H3K27me3 hypermethylated in *ref6* mutants (Paper III – Figure 3). I then wondered whether H2Aub1 could recruit REF6. Previous research showed that REF6 is recruited by CTCTGYTY motifs, but that presence of these motifs were not sufficient to explain REF6 binding. I showed that REF6 was enriched on H2Aub1 peaks and that the CTCTGYTY motifs were necessary for REF6 recruitment, but that the presence of H2Aub1 is associated with increased binding (Paper III – Figure 3). Finally, previous research indicated that REF6 binds genes involved in stimulus response. Our GO analysis showed that genes marked with H2Aub1 but not H3K27me3 were enriched for stimulus response genes (Paper III – Table S7). I furthermore discovered that the expression of genes with H2Aub1 tended to change more frequently in response to stimuli than genes without H2Aub1, or with H3K27me3 (Paper III – Figure S7). Based on this data I created a model in which H2Aub1 serves as a recruiter for PRC2, but also for REF6. Genes with H2Aub1 can therefore be quickly switched between active and repressive states in response to stimuli. Removal of H2Aub1 is thus required for stable repression.

The finding that mammalian BAP1 is an activating H2A de-ubiquitinase has been advanced as evidence that repressive de-ubiquitination does not exist in mammals, and perhaps flies (Campagne *et al.*, 2019). But the model that we

propose allows both repressive and activating H2A de-ubiquitination. If H2Aub1 is removed before PRC2 can be recruited, it will prevent PRC2 recruitment in the future and hence appear to activate the locus. But if H2Aub1 is removed after PRC2 recruitment, H3K27me3 has already been established, and feedback loops (through EED, PC, EMF1c) will ensure this mark is faithfully maintained. In this case removing H2Aub1 will prevent demethylation, and hence prevent reactivation. The timing then makes all the difference, and so it is easily possible that other de-ubiquitinases, even those homologous to BAP1 like CALYPSO, have a different role in the PRC-repressive system.

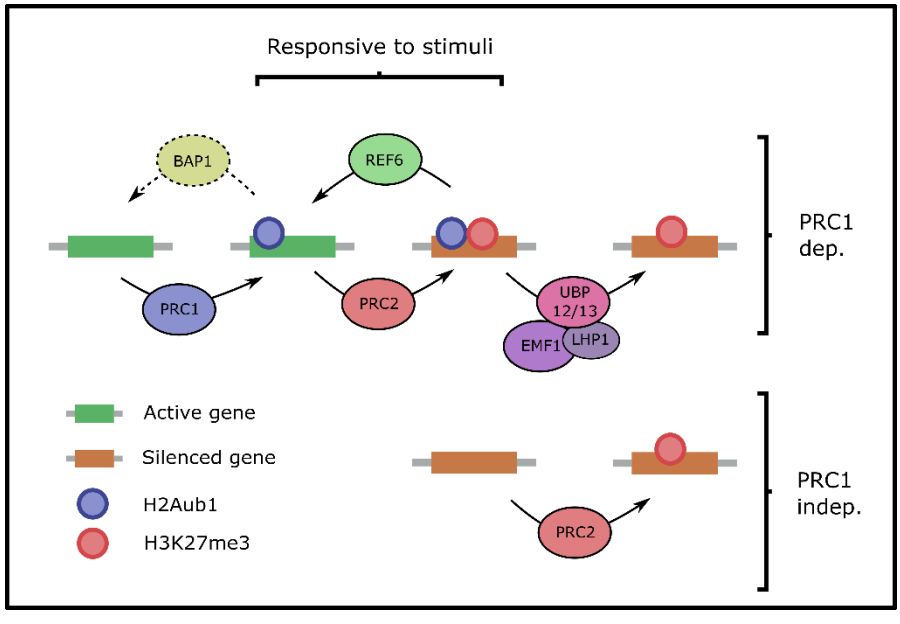

Figure 3. H2A de-ubiquitination is required for stable repression. PRC2 has two modes of recruitment: PRC1-dependent (PRC1 dep.) to active genes (top), and PRC1-indendent (PRC1 indep.) to silenced genes (bottom). PRC1/H2Aub1 can not only aid in PRC2 recruitment, but also in REF6 recruitment. This means that genes carrying H2Aub1 are responsive: they can be quickly switched from active to repressed state and vice versa in response to a stimulus. Stable repression then requires removal of H2Aub1 by UBP12/13. This general mechanism is not incompatible with the finding that de-ubiquitination by BAP1 causes gene activation in mammals, since BAP1 may remove H2Aub1 before PRC2 recruitment. BAP1 is drawn using dashed lines to indicate that this merely a hypothesis, and a BAP1 equivalent has not been identified in plants.

4 Conclusions

1. MSI1 and HDA19, together with SIN3-like proteins and HDC1, form a histone de-acetylase complex that attenuates the ABA-response by de-acetylating ABA-receptors.

2. The early flowering trait of the invasive population of *A. artemisiifolia* is caused by an (over-) dominant genetic factor that likely works by activating the floral activator *FTL1* earlier than normal, and by keeping the floral repressor *FTL2* lowly expressed. Early flowering was accompanied by reduced reproductive output, which is evolutionarily disadvantageous under long vegetation periods. However, under short vegetation periods, only early-flowering plants can produce seeds, making the higher seed set of late-flowering plants irrelevant. I thus conclude that earlier flowering is likely a specific adaptation to the higher latitudes of northern Europe.

3. PRC2 has two modes of recruitment: PRC1-independent recruitment on silent genes, and PRC1-dependent recruitment on active genes. In the second category, H2Aub1 is required for the recruitment of PRC2, but it also allows recruitment of REF6. H2Aub1 thus allows the gene to be switched quickly between active and repressive states. For stable silencing UBP12/13 are required to remove H2Aub1.

5 Future Perspectives

The PRC1/2 repressive system has received much attention, but there are still many gaps in our understanding of its function, especially in plants. In Arabidopsis there are three BMI1-class proteins and two RING1-class proteins, which can form 6 different PRC1 complexes. It is likely that there is some degree of functional divergence, as is the case in mammals, but this has not been studied in detail. The proteins EMF1 and LHP1 are subunits of the EMF1 complex, but considering their many interaction partners it is possible that they are also (accessory) parts of PRC1, PRC2 or other complexes. We hypothesize that UBP12/13 are part of EMF1c because they interact with LHP1 and have a similar function in the maintenance of H3K27me3 like EMF1c, but this requires to be tested. Our finding that MSI1 functions in yet another complex shows the importance of resolving complex compositions, and so reciprocal IP-MS experiments like done for the mammalian PRC1 complexes (Gao *et al.*, 2012) are required to disentangle the mechanisms controlling repressive memory.

Another important aspect to be disentangled is the complexity of the substrates of the methylases, ubiquitinases, and de-ubiquitinases. For instance, in mammals, PRC2 can methylate transcription factor GATA4 to inhibit its function resulting in a repressive output that is not mediated by H3K27me3 (He *et al.*, 2012a). Mammalian RING1B can add branched poly-ubiquitin chains to itself to stimulate its own activity (Lin *et al.*, 2008; Ben-Saadon *et al.*, 2006). USP7 is an eraser of the mono-ubiquitin mark on H2B (Sarkari *et al.*, 2009; van der Knaap *et al.*, 2005), and H3 (Yamaguchi *et al.*, 2017), but also a stabilizer of RING1B by removing the poly-ubiquitin chain (Lecona *et al.*, 2015; de Bie *et al.*, 2010; Maertens *et al.*, 2010), and an inhibitor by removing the activating branched poly-ubiquitin (de Bie *et al.*, 2010). BAP1 not only removes the mono-ubiquitin mark on H2A, but also stabilizes chromatin remodelling complex INO80 (Lee *et al.*, 2014), amongst other things. UBP12/13 have been shown to

be involved in protein stabilization too, in addition to H2A de-ubiquitination (Lee *et al.*, 2019; An *et al.*, 2018; Jeong *et al.*, 2017). This means that in addition to identifying interacting factors and resolving the complexes, we need to study whether the interactors get post-translationally modified, because the functional output that we observe in mutants may not (all) be mediated by the enzymes' canonical function.

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Popular science summary

In multicellular organisms, each cell adopts a specialized function during the course of differentiation. Genes are the blueprints for the characteristics of the cells, but generally do not change. Rather, it is the genetic program that changes: each gene can be switched on or off, and the sum of the activity states of all genes determines the characteristics of the cell. These states are not fixed, and internal or external signals, like hormones and light, may cause some genes to switch. Nevertheless, a certain degree of stability is important. Genes should not be switched on or off because of environmental "noise". Accidental on-switching may cause precocious developmental transitions like flowering during the winter, or seed germination before shedding. And in humans aberrant switching can trigger tumorous growths. To prevent these events from happening, a collection of systems has evolved which are now collectively referred to as "epigenetics". Epigenetics involves modifications of the nucleobase 'letters' in the DNA, as well as modifications of DNA-associated proteins called histones. These modifications together act as an additional code, on top of the genetic code, containing information about whether the gene should be on or off. This thesis focused on repressive mechanisms (i.e. that keep genes off), especially in relation to the process that regulates when plants should flower or mount a stress response. We discovered a new role for the multi-tasked epigenetic factor called MSI1 in the inhibition of the salt-stress response in Arabidopsis. We slightly lifted the veil on the genetic mechanism behind the early flowering trait of a population of common ragweed, and found that this trait allows it to invade northern Europe. And we discovered that one particular histone modification (H2Aub1) is positively associated with the ability for a gene to switch its activity state, and removal of this modification is required for stable repression.

Populair-wetenschappelijke samenvatting

In multicellulaire organismen neemt elke cel een gespecialiseerde functie aan tijdens de loop van het differentiatieproces. Genen zijn de blauwdrukken voor de eigenschappen van de cel, maar veranderen doorgaans niet. Wat er verandert is het genetische programma: elk gen kan aan- en uitgezet worden, en alle aan/uit-standen gecombineerd bepalen de eigenschappen van de cel. Deze standen staan niet vast, en interne en externe signalen zoals hormonen en licht kunnen ervoor zorgen dat een gen omgeschakelt wordt. Desalniettemin, een zekere stabiliteit is belangrijk; genen mogen niet omgeschakelt worden door omgevings-"ruis". Als genen per ongeluk aan gaan dan kunnen ontwikkelingstransities te vroeg plaatsvinden, bijvoorbeeld bloeien tijdens de winter, of ontkiemen van de zaden op de moederplant. En in mensen kunnen zulke verkeerde omschakelingen tumorgroei veroorzaken. Om deze gebeurtenissen te voorkomen is er een collectie van systemen geëvolueerd dat nu collectief "epigenetica" genoemd wordt. De epigenetica omvat modificaties van nucleobasen (letters) in het DNA, maar ook modificaties van DNA-geassocieerde eiwitten genaamd histonen. Deze modificaties werken samen als een extra code bovenop de genetische code, en bevatten informatie over de aan/uit-standen van de genen. Deze thesis richt zich op repressieve mechanismen (die genen op de uit-stand houden), met name zij die regelen wanneer de plant moet bloeien, of reageren op een stressor. Wij ontdekten een nieuwe rol voor een reeds drukke epigenetische factor genaamd MSI1 in de remming van de zoutstressrespons in Arabidopsis. Wij belichtte het genetische mechanisme achter de vroege bloeitijd van een populatie van alsemambrosia, en vonden dat deze eigenschap het mogelijk maakt noord-europa te koloniseren. Daarnaast ontdekten wij dat een bepaalde histonmodificatie (H2Aub1) positief geassocieerd is met de neiging tot omschakelen, en het verwijderen van deze modificatie is noodzakelijk voor stabiele remming.

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