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REVIEW PAPER

Epigenetic regulation of temperature responses: past successes and future challenges

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Abstract

In contrast to animals, plants cannot avoid unfavorable temperature conditions. Instead, plants have evolved intricate signaling pathways that enable them to perceive and respond to temperature. General acclimation processes that prepare the plant to respond to stressful heat and cold usually occur throughout the whole plant. More specific temperature responses, however, are limited to certain tissues or cell types. While global responses are amenable to epigenomic analyses, responses that are highly localized are more problematic as the chromatin in question is not easily accessible. Here we review current knowledge of the epigenetic regulation of *FLOWERING LOCUS C* and *FLOWERING LOCUS T* as examples of temperature-responsive flowering time regulator genes that are expressed broadly throughout the plants and in specific cell types, respectively. While this work has undoubtedly been extremely successful, we reason that future analyses would benefit from higher spatiotemporal resolution. We conclude by reviewing methods and successful applications of tissue- and cell type-specific epigenomic analyses and provide a brief outlook on future single-cell epigenomics.

Keywords: Cell-specific, chromatin, epigenomics, flowering, FLOWERING LOCUS C (FLC), FLOWERING LOCUS T (FT), temperature, tissue-specific, vernalization.

Introduction

Temperature is an environmental factor that strongly influences the growth and development of organisms. This is particularly true for plants, which as sessile organisms cannot evade adverse environmental conditions. Instead, plants have evolved intricate molecular mechanism that enables them to sense and respond to ambient temperature (Capovilla *et al.*, 2015; Hayes *et al.*, 2021). In many plants, traits such as timing of organ initiation and growth rate are particularly susceptible to temperature (Casal and Balasubramanian, 2019). This endows plants with a high degree of phenotypic plasticity. However, there are limits to a plant's capacity to adjust to its environment and numerous studies have demonstrated that

Abbreviations: CME, cold memory element; FACS, fluorescence activated cell sorting; FANS, fluorescence activated nuclei sorting; INTACT, isolation of nuclei tagged in specific cell types; LCM, laser capture microdissection; LD: long day; PcG, Polycomb group; SAM, shoot apical meristem.

temperature can have pronounced effects on the fitness, distribution, and diversity of a species (Atkin *et al.*, 2006; Nicotra *et al.*, 2010; Gil and Park, 2019).

Throughout their life, plants might experience a wide range of different temperatures, from benevolent conditions that support growth to extreme heat or cold. What constitutes heat or cold to a plant is species specific, but for each species one can define cardinal points at which growth ceases because temperatures drop below or exceed a tolerable minimum or maximum. Accordingly, the response of a plant to (changes in) temperature can range from minor adjustments of cellular and physiological processes to shedding of organs or even the death of the whole organism in the hope of more favorable conditions for the next generation (Mittler *et al.*, 2012). Apart from such general effects on plant physiology and fitness, temperature and in particular cold is well known to affect the timing and execution of developmental phase transitions such as seed germination, induction of flowering, and bud break in trees. In contrast to more general heat or cold acclimation processes, temperature often controls these developmental phase transitions in certain tissues or cell-types.

The molecular mechanisms underlying temperature perception in plants are only partially understood. However, plants seem to lack dedicated thermosensors that perceive changes in temperature and orchestrate responses throughout the organism. Instead, the emerging picture is that plants have co-opted diverse factors and signaling pathways to perceive and react to temperature. A core component of temperature signaling in plants involves responses to changes in membrane fluidity (Los *et al.*, 2013). Low temperatures cause a stiffening of the membrane, which leads to calcium influx into the cytoplasm, where it triggers a signaling cascade that eventually results in cold acclimation (Ding *et al.*, 2019). In addition, factors involved in light perception such as phytochrome B (Legris *et al.*, 2016; Jung *et al.*, 2016) and phototropin (Fujii *et al.*, 2017), as well as EARLY FLOWERING 3 (ELF3), a core component of the evening complex of the circadian clock (Jung *et al.*, 2020), and secondary RNA structures (Chung *et al.*, 2020) have been implicated in temperature sensing. Furthermore, temperature-dependent H2A.Z deposition has been suggested to regulate temperature responses, in particular the expression of *FLOWERNG LOCUS T* (*FT*) by PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Kumar and Wigge, 2010; Kumar *et al.*, 2012). Interestingly, SUPPRESSOR OF PHYA-105 (SPA) proteins, best known for their role in light signaling (Hoecker *et al.*, 1998; Laubinger *et al.*, 2004), have recently been shown to regulate the phytochrome B–PIF4 module at high ambient temperature (Lee *et al.*, 2020). Temperature is usually thought to be perceived throughout the plant. However, recently a case of cell autonomous temperature perception and cell specific responses has been reported in Arabidopsis (Bellstaedt *et al.*, 2019). This mechanism seems to be evolutionarily conserved as similar effects were also observed in tomato and cabbage (Bellstaedt *et al.*, 2019). For more detailed information on temperature perception and signaling, there are comprehensive recent reviews that summarize the current state of the field (Casal and Balasubramanian, 2019; Jin and Zhu, 2019; Lin *et al.*, 2020; Hayes *et al.*, 2021).

Ultimately, perception of temperature changes triggers a reprogramming of the transcriptome that not only enables the plant to rapidly acclimate to an acute change in temperature but also initiates the necessary long-term response. For instance, PIF7-mediated activation of a group of high temperature-responsive genes under a warm cycling day temperature suggests how plants acclimate to warm long-day (LD) conditions (Chung *et al.*, 2020). Similarly, *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes have recently been implicated in heat triggered transcriptional reprogramming in reproductive tissue by affecting mainly ABA signaling to provide thermotolerance during the reproductive stage of plant development (Chao *et al.*, 2017). Another recent example demonstrating the widespread effects of temperature on the transcriptome concerns the transcription factors HEAT SHOCK FACTOR A1a (HSFA1a) and circadian clock proteins REVEILLE 4 (RVE4) and RVE8, which have recently been shown to regulate the first wave of heat shock-induced transcriptional reprograming, to regulate the circadian clock and thereby enabling plants to anticipate high temperatures during the day (Li *et al.*, 2019).

Not surprisingly, these transcriptional changes are caused by or at least occur concomitant with changes at the chromatin level (Kim *et al.*, 2015). The basic concept is that genes located in more tightly packed regions of the genome are poorly accessible and hence expressed at a lower level or are completely silenced (Beisel and Paro, 2011; Klemm *et al.*, 2019). Nucleosomes, in which DNA is wrapped around a complex of eight histone proteins, form the fundamental unit of chromatin packaging (Andrews and Luger, 2011). The histone proteins in the nucleosome can be post-translationally modified, for example by adding methyl or acetyl groups or by ubiquitination (Zentner and Henikoff, 2013). Ultimately, these chromatin modifications affect the packaging of the DNA and thereby its accessibility for transcription factor and RNA polymerase binding and function.

Temperature has been shown to affect DNA accessibility by modulating nucleosome positioning, arrangement, and composition. For example, it has been shown that the histone variant H2A.Z, which is incorporated into nucleosomes at the transcription start site, is evicted from chromatin at elevated temperatures, thereby enabling transcription of temperature-regulated genes (Kumar and Wigge, 2010). However, depletion of H2A.Z at warm temperatures is not autonomous but seems to require additional factors such as HSFA1a and possibly other HSFA transcription factors as well as histone deacetylation by HISTONE DEACETYLASE 9 (HDA9) and POWERDRESS (PWR) (Kumar and Wigge, 2010; Cortijo

et al., 2017; Tasset et al., 2018; van der Woude et al., 2019). Apart from nucleosome composition, changes in temperature also have pronounced effects on histone modifications, regulating the expression of thousands of genes.

Regulation of flowering by temperature

A developmental process that is strongly affected by temperature and has been studied in detail is the transition from vegetative growth to reproductive development, or the transition to flowering (Huijser and Schmid, 2011; Posé et al., 2012; Romera-Branchat et al., 2014). The floral induction is controlled by multiple pathways that integrate environmental and endogenous signals (Wils and Kaufmann, 2017). An important environmental signal that regulates flowering in many species is daylength or photoperiod, which is perceived in the leaves. Permissive photoperiod results in the induction of a flower-inducing signal, called florigen, in the phloem companion cells in the leaf vasculature that is subsequently transported to the growing tip of the pant, the shoot apical meristem (SAM), where it triggers the transition to flowering (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Wigge, 2011; Lee and Imaizumi, 2018). FLOWERING LOCUS T (FT) in Arabidopsis and related proteins from other species have been shown to act as evolutionarily conserved florigens (Lifschitz et al., 2006; Tamaki et al., 2007; Wigge, 2011). Even though *FT* expression in Arabidopsis is mainly regulated by photoperiod, ambient temperature has been shown to have a strong effect on *FT* expression, to the point where elevated ambient temperature can induce flowering under otherwise non-inductive short days (Balasubramanian et al., 2006; Capovilla et al., 2015). In addition, many plants, including winter-annual natural accessions of Arabidopsis, require exposure to prolonged periods of cold in order to be able to induce flowering or bud break during the next spring when conditions are favorable (Chouard, 1960). This process is referred to as vernalization. Genetic and molecular analyses have demonstrated that the vernalization response is based on an epigenetic memory of cold. Essentially, during vernalization the expression of floral repressors is epigenetically silenced in response to prolonged exposure of the plant to cold. Importantly, silencing of the repressor is maintained in somatic tissues even after plants are exposed to more benign temperatures. However, the silencing is reset during early embryogenesis to ensure the vernalization requirement in the subsequent generation.

The main target of vernalization in winter-annual accessions of Arabidopsis is *FLOWERING LOCUS C* (*FLC*), which is expressed broadly throughout the plant (Madrid et al., 2021). Over the past years, regulation of *FLC* in response to vernalization has become the best-studied example of epigenetic regulation in plants, and possibly beyond (Whittaker and Dean, 2017). Despite all the progress made, even in the case of *FLC*—and more so for other flowering time genes such as *FT*—questions remain regarding the details of how regulation at the chromatin level mediates the response to temperature.

Here we review current state of knowledge regarding the epigenetic regulation of two important flowering time genes, *FLC* and *FT*, by environmental factors with a focus on temperature. While studies of these two genes have undoubtedly been extremely successful, numerous questions regarding their regulation by temperature still remain. We suggest that one reason for this might be difficulties in performing epigenomic analyses at the tissue specific, cell type specific, or even single cell level and discuss how recent methodological developments may help to overcome or at least alleviate these limitations.

Epigenetic regulation of *FLOWERING LOCUS C*

Winter-annual accessions of Arabidopsis need to be vernalized before flowering can commence in the coming spring. Genetic analyses have identified *FLC* as the main target of vernalization. *FLC* encodes a MADS-domain transcription factor that acts as strong floral repressor. Conceptually, the regulation of *FLC* expression can be divided into four distinct phases (Fig. 1): before cold exposure *FLC* is strongly expressed in the seedling and the vegetative plant; second, *FLC* is silenced in response to cold, and third, the silenced state is maintained in somatic tissues even after plants are returned to warmer temperature; and finally, silencing of *FLC* is reset during reproductive development to ensure high levels of *FLC* expression and the vernalization requirement in the subsequent generation. Over the past two decades, enormous progress has been made in understanding the molecular mechanism underlying the epigenetic regulation of *FLC* (Michaels and Amasino, 1999; Gendall et al., 2001; Levy et al., 2002; Bastow et al., 2004; Sung and Amasino, 2004; Greb et al., 2007; Yuan et al., 2016; Jiang and Berger, 2017; Tao et al., 2019).

Prior to winter cold exposure, FRIGIDA (FRI) and interacting proteins FRIGIDA-LIKE 1 (FRL1), SUPPRESSOR OF FRIGIDA4 (SUF4), FLC EXPRESSOR (FLX), and FRIGIDA-ESSENTIAL 1 (FES1) form a complex (FRIc) that activates *FLC* transcription (Choi et al., 2011; Li et al., 2018*b*). FRIc associates with histone methyltransferases, including the COMPASS-like complex and EARLY FLOWERING IN SHORT DAYS (EFS; SDG8), the histone acetyltransferases HISTONE ACETYLTRANSFERASE OF THE MYST FAMILY 1 (HAM1) and HAM2, UBIQUITIN-CONJUGATING ENZYME 1 (UBC1), as well as the histone H2A.Z-depostion complex SWR1c, the RNA polymerase II (Pol II) associated factor 1 (PAF1c) complex, and the nuclear pre-mRNA cap-binding complex (CBC) to form a FRIsc supercomplex at the *FLC* locus. This causes deposition of active histone marks, H3K4me3 and H3K36me3, resulting in high *FLC* expression, which prevents the floral transition (Choi et al., 2011; Li et al., 2018*b*).

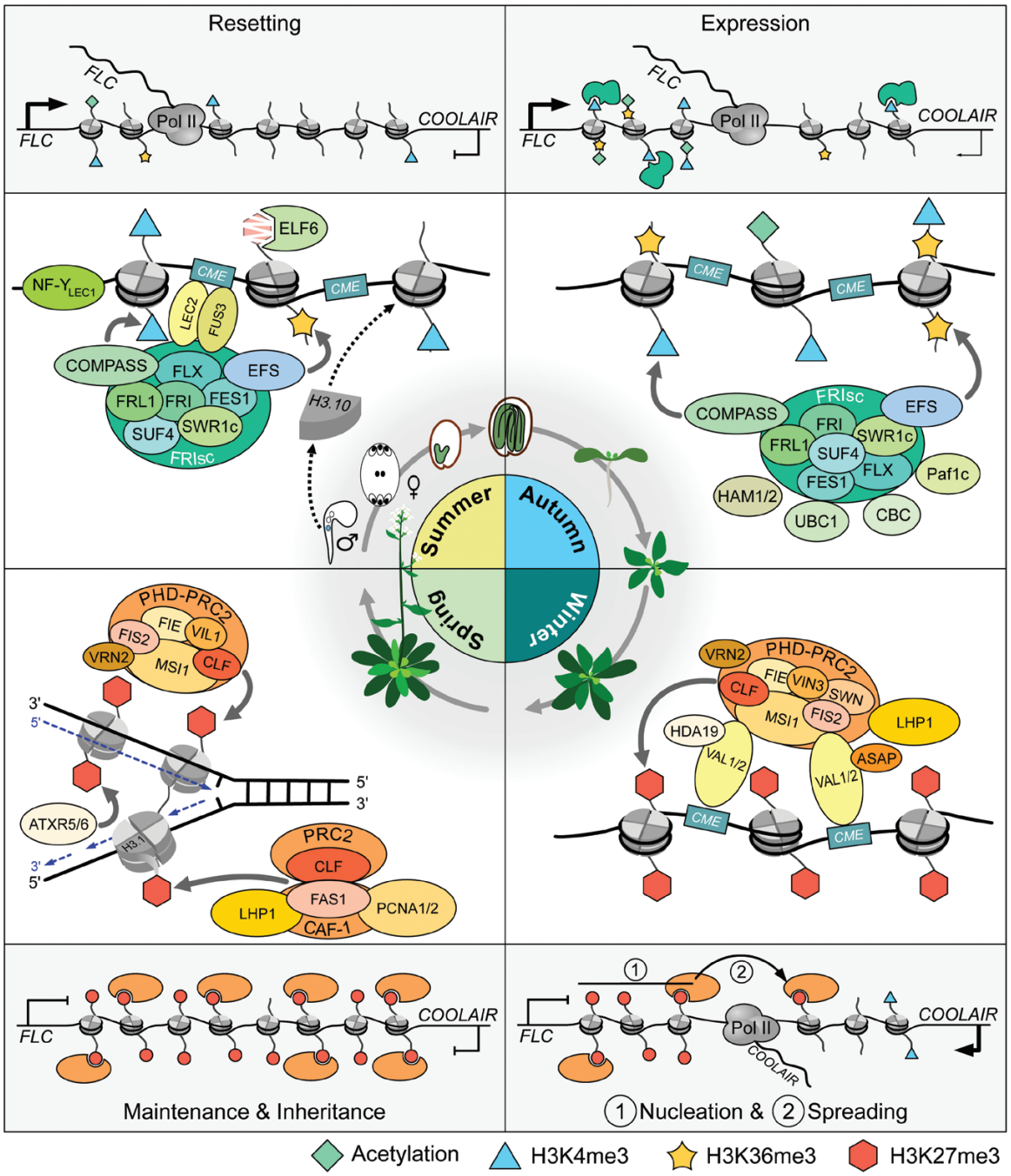


Fig. 1. Regulation of *FLC* expression and chromatin during vernalization. *FLC* is highly expressed in vegetative tissues in winter-annual accessions of Arabidopsis prior to vernalization. Expression of *FLC* is ensured by histone acetylation, as well as deposition of H3K4me3 and H3K36me3 by the FRI supercomplex (FRIsc) and the Compass complex, respectively. Activating marks are removed and replaced by H3K27me3 starting in the nucleation region by the activity of PHD–PRC2 and other proteins in response to winter cold. Silencing spreads from the nucleation region across the *FLC* locus upon return to warm temperatures. Silencing is maintained in the plant after vernalization through mitotic cell divisions through the activity of specific isoforms of PHD–PRC2, the CAF-1 complex, and ATCR5 and ATXR6. Silencing of *FLC* is reset during meiosis and early embryogenesis to ensure the vernalization requirement in the subsequent generation.

Winter cold exposure gradually induces the expression and accumulation of a PLANT HOMEODOMAIN (PHD) protein, VERNALIZATION INSENSITIVE 3 (VIN3) (Sung and Amasino, 2004; Bond *et al.*, 2009). This gradual increase in *VIN3* expression has recently been shown to rely on a NAC transcription factor, NTL8, which slowly accumulates in Arabidopsis as a consequence of the slow growth at low temperatures (Zhao *et al.*, 2020). VIN3 together with its homolog

VIN3-LIKE 1 (VIL1)/VERNALIZATION 5 (VRN5) interacts with a core Polycomb-group repressive complex 2 (PRC2) consisting of CURLY LEAF (CLF), SWINGER (SWN), VERNALIZATION 2 (VRN2), FIE (FERTILIZATION INDEPENDENT ENDOSPERM), and the WD-40 domain protein MULTICOPY SUPRESSOR OF IRA 1 (MSI1) to form a cold-specific PHD–PRC2 complex (Wood *et al.*, 2006; Kim and Sung, 2013; Yang *et al.*, 2017). This cold specific PHD–PRC2 localizes at an intragenic nucleation region, which encompasses three nucleosomes centered over the first exon and part of the first intron of *FLC*, which results in an local increase in H3K27me3 levels, the first step in the silencing process (Finnegan and Dennis 2007; De Lucia *et al.*, 2008; Angel *et al.*, 2011; Yang *et al.*, 2017). However, these initial cold-mediated chromatin modifications are confined to three nucleosomes around the nucleation region and provide only metastable silencing (Angel *et al.*, 2011).

Targeting of PHD–PRC2 to the *FLC* nucleation region was recently shown to depend on specific *cis* regulatory sequences that function as a Polycomb response element, also known as a cold memory element (CME) (Qüesta *et al.*,2016; Yuan *et al.*, 2016). The CME located in the first intron of *FLC* contains two Sph/RY consensus sites that are bound by two transcriptional repressors, VIVIPAROUS1/ABI3-LIKE (VAL1) and VAL2 through their B3 domain. The VAL proteins directly interact with LIKE HETEROCHROMATIN PROTEIN (LHP1), the core PRC2 subunit MSI1, histone deacetylase 19 (HDA19), and apoptosis- and splicing-associated protein (ASAP) complex components and guide the silencing machinery to the nucleation region (Yuan *et al.*, 2016; Qüesta *et al.*, 2016; Xiao *et al.*, 2017; Sasnauskas *et al.*, 2018). Importantly, the CME not only is essential to recruit the VALs and PHD–PRC2 to *FLC* during winter cold exposure but is also required to maintain *FLC* silencing upon return to warm temperature.

Another interesting aspect of *FLC* regulation in response to cold is the contribution of several long non-coding RNAs derived from the *FLC* locus (Swiezewski *et al.*, 2009; Heo and Sung, 2011; Kim and Sung, 2012, 2017; Csorba *et al.*, 2014; Kim *et al.*, 2017; Tian *et al.*, 2019). *COOLAIR* is an antisense transcript derived from *FLC* that is up-regulated during vernalization independently of VIN3. *COOLAIR* transcripts are apparently not essential for vernalization to occur, but seem to be involved in coordinating the switch between H3K36me3 and H3K27me3 at the nucleation region (Swiezewski *et al.*, 2009; Helliwell *et al.*, 2011; Csorba *et al.*, 2014). It has recently been reported that the RNA binding protein FLOWERING CONTROL LOCUS A (FCA), a component of the autonomous flowering time pathway, interacts with CLF, a subunit of PRC2 with histone methyl-transferase activity, and binds nascent *COOLAIR* lncRNA, thereby promoting tri-methylation of H3K27 at *FLC* (Tian *et al.*, 2019). Unlike *COOLAIR*, two other lncRNAs, *COLDAIR* and *COLDWRAP*, are sense transcripts that appear to regulate *FLC* methylation in a more direct manner by direct association with CLF (Heo and Sung, 2011; Kim and Sung, 2017). It seems likely that the CME–VAL1/VAL2 regulatory module in coordination with the lncRNAs *COLDWRAP* and *COLDAIR* functions in recruiting PHD–PRC2 and *FLC* repression. However, the underlying molecular mechanisms have not yet been identified.

Another important phase of the vernalization process occurs after return to warm temperatures when the repressive histone modifications spread from the nucleation region across the entire locus, establishing the mitotically stable transcriptional silencing of *FLC*, which conveys the actual 'epigenetic memory of winter cold' (Yang *et al.*, 2017). Part of this response is regulated by members of the *VIN3* gene family. However, the individual members of this family seem to act at different time points. VIN3 and VIL2 function during the actual cold period, whereas VIL1/VRN5 and VIL3 have been shown to contribute mainly to *FLC* repression after cold (Sung *et al.*, 2006; Greb *et al.*, 2007; De Lucia *et al.*, 2008; Kim and Sung, 2012). Specifically, VIN3 is incorporated in the PHD–PRC2 complex under cold conditions only. In contrast, VIL1 remains associated with PRC2 and participates in spreading H3K27me3 across the *FLC* locus after return to warm temperatures (De Lucia *et al.*, 2008).

Finally, repressive histone marks at *FLC* are maintained throughout mitotic cell divisions. This is accomplished through the activity of the PRC2-independent methyl-transferases ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5 (ATRX5) and ATXR6 or the CAF-1 complex, which ensure the efficient deposition of repressive (H3K27) marks on H3.1 at the replication fork (Jacob *et al.*, 2014; Jiang and Berger, 2017). Specifically, the CAF-1 subunit FASCIATA 1 (FAS1) physically interacts with the DNA replication machinery, PROLIFERATING CELL NUCLEAR ANTIGEN 1 (PCNA1) and PCNA2, as well as with the PRC2 subunit CLF to methylate H3.1 during DNA replication, maintaining H3K27me3 marks on the parental *FLC* chromatin and ensuring deposition of repressive marks on the nucleosomes incorporated into the newly synthesized DNA strand (Jiang and Berger, 2017). In addition, direct interaction of FAS1 with the H3K27me3 reader LHP1 has been shown to be required for the spreading of H3K27me3 at the *FLC* locus after replication under warm conditions (Yang *et al.*, 2017; Jiang and Berger, 2017). Furthermore, LHP1–PRC2 also interacts with ENHANCER OF LHP1 (EOL1), a homolog of the Ctf4 DNA polymerase binding protein, during replication, which contributes to the inheritance of H3K27me3 (Zhou *et al.*, 2017). However, while LHP1 is required for the effective spreading of repressive marks across *FLC*, it is not required for the nucleation of repressive marks during vernalization (Yang *et al.*, 2017). In this context, it is worth noting that the switch from the active to repressed state is a cell-autonomous process and that the gradual quantitative down-regulation of *FLC* observed in response to winter cold reflects the increasing

number of cells in which *FLC* is silenced. As a matter of fact, the memory of winter is stored at *FLC* locally *in cis*, with the consequence that *FLC* can be in a 'mixed' expression state in which one copy in a diploid cell is silenced while the other is actively expressed (Angel *et al.*, 2011; Berry *et al.*, 2015).

Recently, cell-specific analysis of sperm cells revealed that the silenced state of *FLC* imposed by H3K27me3 is actively lifted as well as prevented by the incorporation of H3K27me3-resistant histone H3 variant H3.10, thereby leading to a paternal reset of the *FLC* locus (Borg *et al.*, 2020). Other factors such as the polymerase associated factor (Paf1) complex and the SWR1 complex have been shown to participate in re-establishing *FLC* expression during embryogenesis (Choi *et al.*, 2009; Yun *et al.*, 2011). Similarly, the jumonji (JMJ)-domain-containing H3K27 demethylase EARLY FLOWERING 6 (ELF6) has been shown to be required to achieve full reactivation of *FLC* in the embryo and the growing plant (Crevillén *et al.*, 2014). Furthermore, LEAFY COTYLEDON1 (LEC1), which encodes a subunit of an embryonic pioneer transcription factor, nuclear factor Y (NF-Y), has been shown to promote the establishment of an active chromatin state at *FLC* and activates its expression in the proembryo (Tao *et al.*, 2017). More recently, two B3 domain containing transcription factors, LEC2 and FUSCA3 (FUS3), were shown to compete with VAL1/2 for binding to the CME in *FLC* during embryogenesis, resulting in the disruption of Polycomb-mediated silencing (Tao *et al.*, 2019). In addition, enrichment of LEC2 and FUS3 at *FLC* chromatin results in the recruitment of the FRI complex and associated active chromatin modifiers, which leads to the transcriptional activation of *FLC* (Choi *et al.*, 2011; Tao *et al.*, 2019). Taken together, during gametogenesis and embryogenesis the winter cold memory is actively lifted through the removal of PRC2 repression of *FLC* leading to the suppression of flowering until the next cold period.

Regulation of FLOWERING LOCUS T by temperature

FT has been shown to act as a florigen and convey information to induce flowering from leaves to the SAM in response to inductive day length; its gene is a direct target of FLC (Helliwell *et al.*, 2006; Searle *et al.*, 2006). In addition to photoperiod, temperature also regulates expression of *FT* (Song *et al.*, 2013). The Arabidopsis *FT* promoter is unusually long and harbors several evolutionarily conserved regulatory regions (Fig. 2A) (Takada and Goto, 2003; Adrian *et al.*, 2010). Additional regulatory regions have been mapped to the first intron and the 3′ region of *FT* (Helliwell *et al.*, 2006; Searle *et al.*, 2006). Apart from a proximal promoter close to the transcription start site, a long-distance enhancer 5 kb upstream of the transcription start site has been shown to be essential for activation of *FT* expression in response to LD photoperiod through CONSTANS (CO) (Adrian *et al.*, 2010; Zicola *et al.*, 2019). While three variants of the *FT* promotor can be found in Arabidopsis accessions, the presence of the proximal and distal regulatory elements is conserved, leading to a similar flowering response and highlighting the importance of *FT* regulation (Liu *et al.*, 2014). It has been shown that under an inductive photoperiod the long-distance enhancer is brought into proximity of the core promoter through the formation of a chromatin loop, enabling the expression of *FT* (Fig. 2B) (Tiwari *et al.*, 2010; Cao *et al.*, 2014; Gnesutta *et al.*, 2017). Furthermore, a novel enhancer element located 1 kb downstream of *FT* has been identified and shown to control *FT* expression by coordinating the proximal promoter region in response to photoperiod (Zicola *et al.*, 2019). Whether chromatin looping is also involved in regulating expression of *FT* in response to temperature is currently not clear.

However, that *FT* is regulated by temperature is evident from the observation that in most summer-annual Arabidopsis accessions, even a moderate increase or decrease in ambient temperature triggers activation or repression of *FT* expression, respectively (Balasubramanian *et al.*, 2006; Lee *et al.*, 2007; Kumar *et al.*, 2012). Several MADS-domain transcription factors including FLC, SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS M (FLM) have been shown to form a repressor complex that directly binds to and represses *FT* expression under cool ambient temperature (Lee *et al.*, 2007; Posé *et al.*, 2013; Lutz *et al.*, 2015; Capovilla *et al.*, 2017). Another floral repressor, TEMPRANILLO 2 (TEM2) also has been shown to repress *FT* expression at low ambient temperature under LD conditions (Marín-González *et al.*, 2015). Induction of *FT* expression in response to warmer temperatures has been shown to be directly regulated at the nucleosome level. Histone variant H2A.Z has been shown to be evicted from the +1 nucleosome closest to the transcription start site in response to warmer temperatures, thereby making the *FT* chromatin accessible for binding by the bHLH transcription factor PIF4 (Fig. 2C) (Kumar and Wigge, 2010; Kumar *et al.*, 2012). However, in *Brassica rapa*, elevated temperatures have been shown to lead to reduced *FT* expression and delayed flowering through a mechanism involving H2A.Z, indicating that temperature-dependent expression of *FT* is regulated by the same molecular players but that the underlying molecular mechanisms are wired differently in different species (Del Olmo *et al.*, 2019).

The observed chromatin looping and the contribution of H2A.Z clearly indicate that chromatin-related processes play an important role in regulating *FT* transcription. In addition to these regulatory mechanisms, chromatin modifications have been shown to contribute to and add another layer to the regulation of *FT* expression. For example, PRC2 through its component CLF has been shown to directly interact with *FT* chromatin to catalyse H3K27me3 deposition (Jiang *et al.*, 2008). Importantly, binding of CLF and H3K27me3 deposition at the *FT* locus seem to antagonize NF-Y and CO binding and

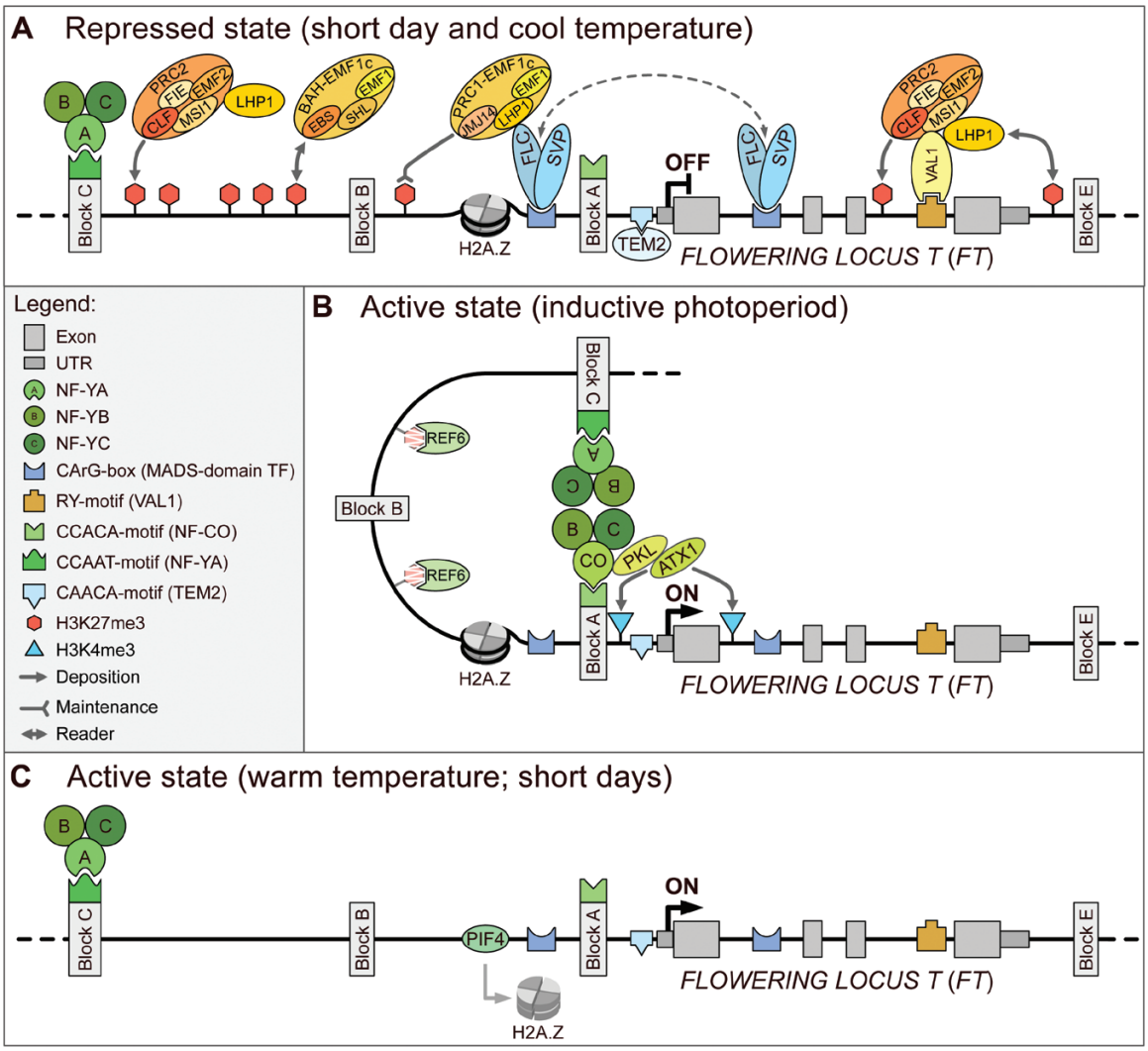


Fig. 2. Epigenetic and transcriptional regulation of *FLOWERING LOCUS T* (*FT*). (A) Expression of Arabidopsis *FT* is suppressed under non-inductive environmental conditions such as short days and/or cool ambient temperature by the combined activity of Polycomb group (PcG) protein complexes that deposit, read, and maintain repressive histone modifications such as H3K27me3, and transcriptional repressors such as FLC, SVP, and TEM2. (B) Upon exposure to inductive photoperiod (long day), repressive histone modifications are removed by the activity of histone demethylases such as REF6. CO protein is stably induced at the end of the day and interacts with NF-YB and NF-YC protein to form a NF-Y–CO complex that binds to an evolutionarily conserved region containing a CCACA motif close to the transcription start site (block A). NF-Y–CO interacts with an NF-Y complex bound to a CCAAT motif in a distal evolutionarily conserved region (block C), resulting in the formation of a chromatin loop. Binding of CO also results in the recruitment of histone modifying enzymes such as PKL and ATX1 and the deposition of activating chromatin modifications such as H3K4me3 to the *FT* core promoter and gene body. (C) Warm ambient temperature induces the eviction of H2A.Z-containing nucleosomes from the *FT* locus close to the transcription start, enabling binding of the PIF4 transcription factor. In addition, warm temperatures result in the down-regulation and/or degradation of MADS-domain floral repressors such as FLC and SVP, thereby indirectly facilitating flowering.

thereby prevent chromatin looping and *FT* expression in the late afternoon or near dusk (Cao *et al.*, 2014; Liu *et al.*, 2018; Luo *et al.*, 2018). Similarly, a PRC1-like complex consisting of EMF1, LHP1, and the H3K4 demethylase JMJ14 ensures repression of *FT* before dusk and at night to prevent photoperiod-independent flowering. However, binding of this repressive complex to *FT* chromatin is disrupted by photoperiodic activity of CO at dusk (Calonje *et al.*, 2008; Wang *et al.*, 2014). Importantly, JMJ14 is not the only demethylase to regulate FT chromatin (Lu *et al.*, 2010). Two other EMF1-interacting H3K4 demethylases, JMJ15 and JMJ18, have also been linked to regulate Polycomb group (PcG)-mediated *FT* repression (Yang *et al.*, 2012*a*, *b*). Additionally, the H3K27me3 demethylase JMJ13 has been shown to act as a temperature- and photoperiod-dependent repressor of flowering (Zheng *et al.*, 2019). In contrast, overexpression of the JMJ domain-containing histone H3K27me3 demethylase RELATIVE OF EARLY FLOWERING 6 (REF6) results in the activation of *FT* transcription (Lu *et al.*, 2011). More recently it was shown that two homologs of the bromo-adjacent homology (BAH)

domain containing proteins, EARLY BOLTING IN SHORT DAY (EBS) and SHORT LIFE (SHL), interact with EMF1 and function as H3K27me3 readers, indicating that this complex performs PRC1-like roles in implementing Polycomb silencing of FT (Li *et al.*, 2018*a*; Yang *et al.*, 2018).

The role of PcG as a repressor of gene expression is antagonized by Trithorax-group (TrxG)-like proteins that promote transcription. In the case of *FT*, repression by PcG proteins is opposed by the chromatin-remodeling factor PICKLE (PKL), which recruits the TrxG-like H3K4me2/3-specific methyl transferase ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1) to establish active marks specifically around dusk (Jing *et al.*, 2019*b*). In addition, interaction between CO and PKL has been reported to enhance the access of CO to the CO-responsive elements in the proximal region of the *FT* promoter, thereby enhancing the recruitment of NF-Y–CO at *FT* to promote floral transition (Jing *et al.*, 2019*c*). However, little else is known about the mechanism behind establishing active marks at *FT*.

There exist several interesting similarities and points of convergence between the epigenetic regulation of *FLC* and *FT*. For example, VAL1 has recently been shown to bind to intronic *cis*-regulatory RY elements in the *FT* locus to which it recruits LHP1 and MSI1 to ensure H3K27me3 deposition to repress *FT* expression before dusk and at night (Jing *et al.*, 2019*a*). While the time scale is quite different, this is nevertheless reminiscent of the role of VAL1 in establishing silencing of *FLC* in response to winter cold. Furthermore, FLC has been shown to bind to the *FT* promoter to regulate flowering time in response to vernalization and changes in ambient temperature (Helliwell *et al.*, 2006; Searle *et al.*, 2006). Surprisingly, the function of these two genes seems to be reversed during the establishment of seed dormancy with FT regulating *FLC* expression and chromatin state by activating *FLC* antisense transcription, suggesting that FT plays a crucial role in integrating maternal temperature history to control dormancy in the seeds (Chen and Penfield, 2018). Furthermore, and similar to the situation in *FT*, chromatin looping has recently been described at the *FLC* locus (Gagliardi and Manavella, 2020).

A need for cell-specific investigations of temperature responses

As discussed above, the past years have seen enormous advances in the field of plant temperature response in respect to the epigenetic regulation of flowering time. However, most of our knowledge originates from analysis of whole seedlings and complex tissues. While undeniably capable of unravelling global effects of temperature on the plant as well as identifying the major players involved in the response, it is not unlikely that such approaches, due to a lack of spatial and temporal resolution, might overlook tissue- or cell type-specific regulatory mechanisms that fine-tune temperature responses.

FLC is the central player of the vernalization-dependent flowering pathway and being the plant gene studied in most detail at the epigenetic level makes a good case for a cell-specific approach. One reason why past analyses of *FLC* were so successful is that *FLC* is expressed rather broadly throughout the plant and that vernalization acts in the majority of cells and tissues, which facilitates chromatin-related studies. Nevertheless, even for the case of *FLC*, many questions remain. For example, it has been shown that the *FLC*-mediated memory of winter is established and stored *in cis* (Angel *et al.*, 2011, 2015; Berry *et al.*, 2015; Rosa *et al.*, 2016), but the precise spatiotemporal events that govern this process are still not fully understood. In contrast, the most prominent direct targets of FLC, *FT* and *SOC1* (Helliwell *et al.*, 2006; Searle *et al.*, 2006; Deng *et al.*, 2011), are expressed in clearly defined cell populations (Samach *et al.*, 2000; Takada and Goto, 2003). *FT* expression is spatially restricted to the phloem companion cells in the minor veins of the leaves, which complicates chromatin-level studies. Nevertheless, as outlined above, enormous progress has been made in understanding the transcriptional regulation of *FT* expression in response to environmental stimuli. However, one is left to wonder if the fact that *FT* expression is only activated in a small number of cells in the phloem might not conceal certain regulatory mechanism from epigenomic analyses conducted in complex tissues.

An example not related to the regulation of flowering time that demonstrates the need for cell-specific (epi-) genomic studies is thermomorphogenesis, which enables plants to adapt their morphology to elevated ambient temperature (Quint *et al.*, 2016). Elongation of the hypocotyl in response to temperature is a well-documented thermomorphogenic response that involves auxin biosynthesis, signaling, and the bHLH transcription factor PIF4 (Gray *et al.*, 1998; Franklin *et al.*, 2011; Sun *et al.*, 2012; Bellstaedt *et al.*, 2019). *PIF4* is most strongly expressed in the leaf vasculature, but to regulate hypocotyl elongation during thermomorphogenesis requires epidermal expression (Kim *et al.*, 2020). How the spatiotemporal expression of *PIF4* in response to temperature is regulated is not fully understood, again highlighting the need for cell- or at least tissue-specific analyses.

Methods for the enrichment of specific cell types

Manual dissection has been used successfully for the enrichment of tissues and tissue-specific epigenomic and gene expression analyses (Schmid *et al.*, 2005; Ma *et al.*, 2005; Lafos *et al.*, 2011; Widman *et al.*, 2014). However, the spatiotemporal resolution that can be achieved using manual tissue dissection is limited. Fortunately, methods have been developed to increase the resolution and mitigate the problem that analyses conducted in complex tissues can obscure epigenetic and gene regulatory mechanisms specific to small populations (Fig. 3).

One strategy is to reduce the complexity of the tissue of interest. This can be achieved using cell cultures that have a high degree of uniformity and synchronicity (Menges and Murray, 2002) or mutants in which certain cell types overproliferated. An example of the latter is the *ap1 cal* double mutant, which displays an enlarged SAM, enabling the sampling of highly enriched meristematic tissue (Bowman *et al.*, 1993). Additionally, such simplified systems can be converted into inducible systems in which cell differentiation can be investigated (Milioni *et al.*, 2002; Wellmer *et al.*, 2006). A potential disadvantage of cell cultures is that the cells are taken out of their tissue context and cultured through many passages and might thus exhibit unusual properties. However, cell cultures are well suited for treatments with chemical inducers (e.g. nutrients, hormones) or environmental stimuli (e.g. light, temperature) to induce responses in a highly synchronized manner.

The second strategy differs from the first in that it relies on the technical isolation of single cells, nuclei, or cell populations from complex tissues that can then be used as input material in subsequent analyses. These techniques include laser capture microdissection (LCM), fluorescence activated cell/nuclei sorting (FACS/FANS) and isolation of nuclei tagged in specific cell types (INTACT).

LCM is a significant step up from manual dissection in respect to resolution of the collected material. In short, areas on fixed and sectioned tissue can be isolated selectively, by either laser assisted attachment on carrier film (Emmert-Buck *et al.*, 1996) or laser dissection and sampling (Schütze and Lahr, 1998). The possibility to isolate cells by visual inspection has the advantage that it can be utilized with any kind of tissue without the need of dedicated transgenic lines. LCM was adopted for plants early on and enabled the tissue-specific investigation of gene expression (Asano *et al.*, 2002; Kerk *et al.*, 2003; Nakazono *et al.*, 2003). While LCM has also been employed successfully in the identification of tissue-specific DNA methylation (Lin *et al.*, 2017), we are not aware of studies that report profiles of epigenomic marks based on LCM. Presumably, issues caused by the fixation of samples and the fact that thin sections often do not contain (intact) nuclei limits the suitability of LCM for epigenomic studies.

FACS has been developed in order to separate and isolate cells from complex mixtures (Bonner *et al.*, 1972). However, it took the combination of tissue-specific fluorescent protein expression and efficient isolation of protoplasts to enable the isolation of specific plant cells using FACS (Birnbaum *et al.*, 2003). FACS facilitates the isolation of the whole cell including RNA, chromatin, and metabolites and thereby potentially enables the widest range of downstream applications (Birnbaum *et al.*, 2003; Zhang *et al.*, 2005; Petersson *et al.*, 2009). However, protoplast isolation requires fresh living tissue, which restricts the sampling and sample processing. Additionally, since the process of cell wall digestion takes the still active cell out of its context, the physiology (and gene expression) of the cell after the treatment could be altered (Davey *et al.*, 2005). To mitigate these problems, FANS has been developed (Zhang *et al.*, 2008). During FANS, nuclei marked by the cell-specific expression of a H2A fluorescent protein fusion (or other highly abundant nuclear localized reporters) are extracted and isolated using a fluorescence activated cell sorter (Zhang *et al.*, 2005, 2008). The advantage of utilizing FANS is that the isolation of nuclei can be done from flash-frozen material, which separates sampling from sample preparation and in addition interrupts cellular processes that could influence gene expression, DNA methylation, or histone modification. Therefore, FANS in theory should deliver a better snapshot of the state of the nucleus *in vivo*.

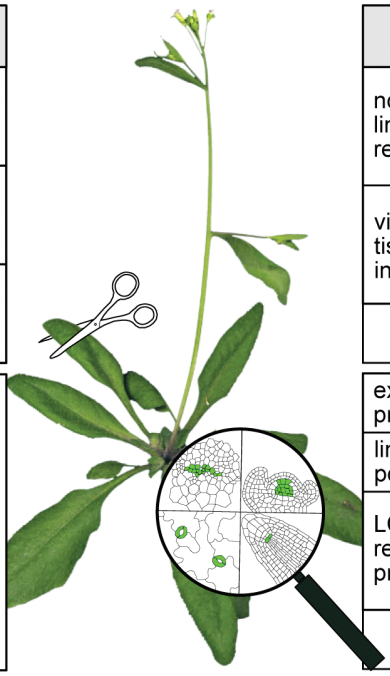
Both LCM and FACS/FANS rely on specialized equipment, and therefore high acquisition and maintenance costs must be considered when working with these methods. In contrast, INTACT has been developed to isolate nuclei from specific cell types without the demand of such equipment (Deal and Henikoff, 2010). In short, INTACT relies on the use of double transgenic plants that express two constructs: a so-called nuclear targeting fusion (NTF) protein, which is expressed from a tissue- or cell type-specific promoter and associates to the nuclear membrane, and a ubiquitously expressed biotin ligase, which can biotinylate the NTF protein *in planta*. Tagged nuclei can then be isolated by affinity purification using streptavidin-coupled beads enabling similar downstream applications to FANS. A comparison of the pros and cons the mentioned methods is shown in Fig. 3A. In summary, several strategies for cell-specific investigations exist in the form of reducing complexity or isolating cells or nuclei. However, due to their flexibility and wide range of possible downstream analyses, strategies involving the isolation of specific cells or nuclei from complex tissues have been adopted most successfully. Except for cell cultures, all of these methods have in common that they usually yield only a relatively small numbers of cells or nuclei and thus input material (nucleic acids) suitable for subsequent analyses.

Tissue- and cell type-specific '-omics' approaches

FACS/FANS and INTACT based methods have been successfully used to isolate cells and/or nuclei from the epidermis, guard cells, phloem companion cells, mesophyll cells, root hair and non-hair cells, root tip and quiescent centre, SAM, microspore, sperm and vegetative cell, endosperm, and embryo (Birnbaum *et al.*, 2003; Nawy *et al.*, 2005; Deal and Henikoff, 2010; Borges *et al.*, 2012; Moreno-Romero *et al.*, 2016; Palovaara *et al.*, 2017; You *et al.*, 2017; Sijacic *et al.*, 2018; Lee *et al.*, 2019; You *et al.*, 2019; Tian *et al.*, 2021; Zheng and Gehring, 2019). Most of these studies reported tissue- or cell type-specific transcriptomes, but only a few studies focused on or included epigenetic approaches. However, all reports on cell-specific approaches have in common that at least some of

A

	Manual dissection	LCM	FACS/FANS	INTACT	
Pros	no special requirements	no dedicated lines/plants are required	RNA, chromatin and metabolites are accessible	no specialized equipment required	Pros
	standard procedures		FANS samples can be frozen before isolation		
	low expense	visual selection of tissue or cells of interest	gateway to current single cell/nuclei pipelines	samples can be frozen before isolation	
		cell specific analysis			
Cons	readout is limited to complex tissues	extensive sample preparation	cell/nuclei marker lines required	dedicated INTACT lines are required	Cons
		limited epigenomic potential	cellsorter is required		
		LCM system and respective proficiency needed	protoplast isolation limits sample processing	bead bound nuclei can interfere in future single nuclei applications	
		specialized low input downstream protocols are needed			



B

Tissue	Method	Dataset generated	Application	Literature
Epidermis	INTACT	DNA accessibility profile and transcriptome	dynamic	Tian et al. 2021
Endosperm	INTACT	H3K9me2/H3K27me1/H3K27me3 and DNA methylation profiles	maternal/paternal comparative	Moreno-Romero et al. 2016
	INTACT	H3K27me3 profile	maternal/paternal comparative	Zheng and Gehring et al. 2019
Embryo Cell populations	INTACT	transcriptome	comparative	Palovaara et al. 2017
Guard cells	FACS	H3K4me3/H3K27me3 profiles and transcriptome	comparative	Lee et al. 2019
Mesophyll cells	INTACT	DNA accessibility profile	comparative	Sijacic et al. 2018
Phloem companion cells	INTACT	H3K4me3/H3K27me3 profiles and transcriptome	dynamic	You et al. 2019
	INTACT	DNA accessibility profile and transcriptome	dynamic	Tian et al. 2021
Root cells	INTACT	H3K4me3/H3K27me3 profiles and transcriptome	comparative	Deal and Henikoff 2010
	FANS	single nuclei DNA accessibility profiles and transcriptomes	comparative	Farmer et al. 2021
Sperm cell and vegetative nucleus	FACS	DNA methylation profile	comparative	Calarco et al. 2012
	FACS	H3.10/H3K4me3/H3K27me3/ H3K27me1/H3K27ac profiles	comparative	Borg et al. 2020
Shoot apical meristem	INTACT	H3K4me3/H3K27me3 profiles and transcriptome	dynamic	You et al. 2017
	INTACT	DNA accessibility profile	comparative	Sijacic et al. 2018
	FANS	DNA methylation profile and transcriptome	dynamic	Gutzat et al. 2020

Fig. 3. Methods for and recent advances in cell-specific studies in Arabidopsis. (A) Comparison of procedures suitable for the isolation of cells or nuclei from plant tissues. INTACT, isolation of nuclei tagged in specific cell-types; FACS, fluorescence activated cell sorting; FANS, fluorescence activated nuclei sorting; LCM, laser capture microdissection. (B) Recent studies advancing cell type-specific epigenomic analysis in Arabidopsis.

their findings would have been overlooked if the experiments had been conducted in complex tissues.

For example, focusing on epigenetic inheritance, Moreno-Romero *et al.* (2016) successfully applied INTACT on endosperm nuclei. Using histone chromatin immunoprecipitation (ChIP)-seq and bisulfite-seq the authors showed that in endosperm the maternal genome contains specific repressive H3K27me3 marks that overlap with DEMETER (DME)-mediated hypomethylated regions (Moreno-Romero *et al.*, 2016). FACS has been successfully used to isolate sperm cells and vegetative cell from pollen (Calarco *et al.*, 2012). DNA methylation and H3K27me3 profiles from these cell types showed that DME-based demethylation is limited to the vegetative cell, whereas in sperm cells active removal and prevention of H3K27 methylation reshapes the epigenome (Calarco *et al.*, 2012; Borg *et al.*, 2020). Furthermore, by combining INTACT and the assay for transposase-accessible chromatin (ATAC)-seq, Sijacic *et al.* (2018) showed that while cells in the SAM and mesophyll cells share most transposase hypersensitive sites, each cell type also features very specific transposase hypersensitive sites, which can be used to predict transcriptional regulatory networks.

These studies have in common that they investigate (and compare) epigenetic features of specific cell types under stable conditions. While clearly very informative, we believe that the real power of tissue- or cell type-specific '-omics' approaches resides in the combined analysis of the dynamic changes of epigenomic features and transcriptomes of the plant in response to perturbations. While we are not aware of any such studies addressing the responses to (changes in) temperature, several recent studies report the dynamic nature of chromatin-related features and transcriptomes in response to acute changes in photoperiod.

For example, by performing ATAC-seq on chromatin isolated using INTACT from the epidermis and phloem companion cells of plants that had been shifted from short-day to LD conditions, Tian *et al.* (2021) recently reported that binding sites of flowering-related transcription factors were enriched in LD-responsive transposase hypersensitive sites and that this enrichment was more prominent in the phloem companion cells than in the epidermis. Similarly, You and colleagues employed INTACT to investigate gene expression and H3K4me3/H3K27me3 dynamics in the SAM and the phloem companion cells in response to a shift in day length and reported combinations of epigenomic markings in these cell types not apparent in complex tissues (You *et al.*, 2017, 2019). More recently, FANS has been employed to monitor gene expression and DNA methylation of the SAM stem cell niche throughout development, indicating that dynamic DNA methylation might contribute to resetting of transposon silencing and early germ cell differentiation (Gutzat *et al.*, 2020). These recent reports indicate that epigenetic properties and responses are intrinsically linked with cell identity, arguing for a more widespread use of tissue- and cell type-specific approaches in '-omics' studies.

Even though far from routine, single cell RNA-seq is now possible in plants and has been applied to compare the transcriptome between and within different cell types, and in response to exogenous stimuli (Jean-Baptiste *et al.*, 2019; Ryu *et al.*, 2019; Shulse *et al.*, 2019; Long *et al.*, 2021; Xu *et al.*, 2021; Zhang *et al.*, 2021). Complementary approaches referred to as 'spatially resolved transcriptomics' combine transcriptomics with *in situ* localization, thereby providing information on the spatial expression of genes in complex tissues (Giacomello *et al.*, 2017; Salmén *et al.*, 2018; Rodriques *et al.*, 2019). In contrast, single cell chromatin studies remain challenging, mostly because of the sparsity of signals that can be obtained from chromatin, which is much lower than that of mRNA. However, accessibility of chromatin in individual plant cells using scATAC-seq has recently been reported (Farmer *et al.*, 2021). Recent examples of cell-specific approaches, the tissues used, and the datasets generated can be seen in Fig. 3B.

Conclusion and future perspectives

Temperature affects plant physiology, growth, and development in manifold ways. Many of these general responses occur in tissues throughout the plant. Other, more specific responses, such as thermomorphogenesis and regulation of developmental phase transitions, are spatially more restricted. While (epi-) genomic and transcriptomic analyses conducted on complex tissues, within limits, are capable of unravelling the gene regulatory networks underlying global responses, tissue- and cell type-specific analyses are needed to understand how changes in ambient temperature shapes plant morphology and affects its fitness. In order to truly understand the gene regulatory processes that govern temperature responses, or responses to other endogenous and environmental cues, analyses will need to be conducted at the single cell level. Fortunately, the methods to perform such cell type-specific analyses are now becoming available in plants. Single cell RNA-seq, while far from routine, is becoming more and more widespread in plants, and single cell ATAC-seq has already been implemented in plants (Farmer *et al.*, 2021). Furthermore, current developments in the animal field such as single cell ChIP-seq (Grosselin *et al.*, 2019), single cell calling card-based transcription factor binding site detection (Moudgil *et al.*, 2020), and single cell DNA adenine methyltransferase identification (Markodimitraki *et al.*, 2020) do show that other types of single cell epigenomic studies are feasible and on the rise.

Another important issue that will need to be taken into consideration in the future is that so far most epigenomic studies in plants have been conducted under highly artificial constant light and temperature regimes. Such conditions, while easy to set up in the lab, are of course not representative of the fluctuating environments that plants are exposed to in nature. It thus seems likely that we underestimate the complexity and dynamic nature of epigenetic regulation in response to environmental stimuli in plants. The feasibility

and benefits of epigenomic studies under fluctuating environmental conditions was recently demonstrated (Nishio *et al.*, 2020), and we will hopefully see more studies such as this in the future.

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