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Towards a better understanding of dormancy in sweet cherry floral buds using molecular and epigenetic approaches

Noémie Vimont

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Centre Mondial de l'Innovation
Nutrition Végétale, Nutrition et Bien-être Animal



université
de **BORDEAUX**



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Par : **Noémie VIMONT**

Towards a better understanding of dormancy in sweet cherry floral buds using transcriptomic and epigenetic approaches

(Compréhension transcriptomique et épigénétique de la dormance chez
des bourgeons floraux de cerisier doux (*Prunus avium*, L))

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Membres du jury :

M. MAURY Stéphane, Professeur, Université d'Orléans
M. FOUCHER Fabrice, Directeur de Recherche, UMR IRSH, Angers
M. PLOMION Christophe, Directeur de Recherche, UMR Biogeco, Bordeaux
Mme ORSEL-BALDWIN Mathilde, Chargée de Recherche, UMR IRSH, Angers
Mme TEYSSIER Emeline, Maître de conférence, Université de Bordeaux
M. ARKOUN Mustapha, Chef de projet, Groupe Roullier-C.M.I, St Malo
Mme DIRLEWANGER Elisabeth, Directrice de Recherche, UMR BFP, Bordeaux

Président
Rapporteur
Rapporteur
Examinateur
Examinateur
Invité
Directrice de thèse

A la mémoire de mon oncle Claude.

Résumé

Compréhension transcriptomique et épigénétique de la dormance chez des bourgeons floraux de cerisier doux (*Prunus avium*, L)

Le réchauffement climatique est déjà une réalité et une hausse des températures de 2 à 4°C est attendue d'ici 2050. Pour les espèces fruitières, un des impacts les plus notables dus au changement climatique a été observé pour la phénologie de la floraison et les dates de maturation des fruits. En effet, la phénologie de la floraison est sous la dépendance directe d'une sortie adéquate de dormance des bourgeons, largement contrôlée par la température. Au cours de mon projet de thèse, des approches physiologiques, moléculaires et transcriptomiques ont été combinées afin d'analyser finement la réponse à la température chez différentes variétés de cerisier ayant des dates de sortie de dormance et de floraison différentes. Les analyses du transcriptome par le biais de RNA-seq ont permis d'obtenir une liste de gènes marqueurs impliqués dans la progression de la dormance et pourront être utilisés comme outil pour l'aide à la décision afin d'estimer le statut de dormance des arbres. Ce qui est particulièrement important pour les producteurs qui ont besoin de connaître un interval temps précis pour appliquer des produits de levée de dormance. Des quantifications de phytohormones ont révélé l'importance de l'acide abscissique et de ses catabolites dans l'induction et la maintenance de la dormance. En complément de l'étude transcriptomique, une approche épigénétique a été faite sur des bourgeons floraux de cerisier. Nous avons trouvé que la sortie de dormance était associée à certaines marques histones comme la méthylation et l'acétylation des histones. L'impact agronomique de ce projet sera d'apporter une meilleure connaissance des gènes et des mécanismes impliqués dans la dormance dans le but de créer de nouvelles variétés adaptées aux futures conditions climatiques en France et dans plusieurs régions du monde.

Mots clés: Dormance, RNA-seq, ChIP-seq, transcriptome, *Prunus avium* L.

UMR 1332 – Biologie du Fruit et Pathologie (BFP)

Equipe : Adaptation du Cerisier au Changement Climatique

71 avenue Edouard Bourlax, 33883 Villenave d'Ornon

Abstract

Towards a better understanding of dormancy in sweet cherry floral buds (*Prunus avium* L.) using molecular approaches

Plants are sessile organisms and must adapt their physiology to seasonal changes to subsist over time. Their ability to perceive and respond to changes in temperature is a key determinant of their survival. For perennial plants, this is especially important because flowering occurs in the next season and reproductive structures can be exposed to high temperature fluctuations, particularly low winter temperatures. One of the strategies to survive under low temperatures is a period of dormancy. In sweet cherry (*Prunus avium* L.), which is a perennial fruit tree belonging to the *Rosaceae* family, dormancy is mainly induced by low temperatures. However, the mean surface temperature of the earth is increasing and this climatic change may have serious negative consequences on dormancy release, potentially resulting in lower cherry production. Despite the effect of temperature on dormancy and bud break, the molecular events regulating these processes are still poorly understood. We performed transcriptomic analysis on flower buds of different cherry cultivars displaying contrasting flowering times to identify genes involved in dormancy regulation. A time-course spanning the entire bud development until flowering was performed to explore specific biological processes. A list of marker genes associated with dormancy progression was identified and can be used as a decision-making tool to estimate the dormancy status of buds. This is particularly relevant for growers who need to know the optimal timing to apply dormancy release products. Phytohormone quantifications have revealed that abscisic acid and its catabolites play an important role in the induction and maintenance of dormancy. In addition, we investigated potential epigenetic mechanisms regulating dormancy using ChIP-seq and epigenetic drugs. We found that histone deacetylation is involved in bud break as well as repressive and active histone marks such as H3K27me3 and H3K4me3, respectively. These results provide a source of information to create predictive models, which will be powerful tools to assist breeding strategies.

Keywords: Dormancy, RNA-seq, ChIP-seq, transcriptome, *Prunus avium* L.

UMR 1332 – Biologie du Fruit et Pathologie (BFP)

Team : Adaptation du Cerisier au Changement Climatique

71 avenue Edouard Bourlaux, 33883 Villenave d'Ornon

Scientific communications

Publications

- N. Vimont**, D. Schoepfer, F. Roudier, E. Dirlwanger, P. A. Wigge, B. Wenden, S. Cortijo (2018) Chromatin immunoprecipitation-sequencing and RNA-sequencing for complex and low-abundance tree buds. *Plant Methods* (in revision).
- N. Vimont**, A. Schwarzenberg, M. Domijan, R. Beauvieux, M. Arkoun, J.C Yvin, S. Cortijo, P.A. Wigge, E. Dirlwanger, B. Wenden (2018) Hormonal balance finely tunes dormancy status in sweet cherry flower buds. *eLife* (in revision).
- N. Vimont**, D. Schoepfer, M. Fouché, V. Charoensawan, J.C. Yvin, M. Arkoun, E. Dirlwanger, S. Cortijo, P.A. Wigge, B. Wenden (2018) Cherry bud developmental phases can be inferred from their transcriptional profiles. (in preparation).
- N. Vimont**, F.X. Quah, M. Arkoun, J.C. Yvin, E. Dirlwanger, P.A. Wigge, B. Wenden & S. Cortijo (2018) H3K4me3 and H3K27me3 modifications are associated with dormancy progression in sweet cherry (*Prunus avium* L.) flower buds. (in preparation).
- N. Vimont**, R. Beauvieux, J.C. Yvin, M. Arkoun, E. Dirlwanger, S. Cortijo, P.A. Wigge, B. Wenden (2017). The presence of H3K4me3 histone mark is positively correlated with expression at the DAM loci in sweet cherry during dormancy. *Acta Horticulturae* (in press) (see the publication in appendix).
- N. Vimont**, R. Beauvieux, A. Schwarzenberg, P.A. Wigge, E. Dirlwanger, B. Wenden (2018). Hormonal control of dormancy in sweet cherry flower buds. *Acta Horticulturae* (in press) (see the publication in appendix).

Presentations

- N. Vimont**, M. Arkoun, J.C. Yvin, P.A. Wigge, E. Dirlwanger, S. Cortijo, B. Wenden (2015) Epigenome dynamics during dormancy in sweet cherry. The 4th European Workshop on Plant Chromatin (EWPC), 25th-26th June 2015, Uppsala (Sweden). **Oral presentation.**
- N. Vimont**, M. Arkoun, J.C. Yvin, P.A. Wigge, E. Dirlwanger, S. Cortijo, B. Wenden (2016) Molecular and epigenetic mechanisms during dormancy in sweet cherry flower buds. The 27th International Conference on Arabidopsis Research (ICAR), 29th June- 03rd July 2016, Gyeongju (South-Korea). **Poster.**
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2017: Award for the best poster at Journées Jeunes Chercheurs BAP in Bordeaux (France).

2017: ISHS student award for the best oral presentation at 8th International Cherry Symposium in Yamagata (Japan).

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List of Figures

Figure number	Figure title	Page number
1.1	Global warming projection	2
1.2	Climate change will depress agricultural yields in most countries in 2050	3
2.1	Cherry taxonomy	5
2.2	Distribution map of wild cherry (<i>Prunus avium</i> L.)	7
2.3	French production of sweet cherry (<i>Prunus avium</i> L.) in 2016 and 2017	10
2.4	Branching pattern along a cherry annual branch	11
2.5	Picture of a cherry spur	12
2.6	Cherry flower development stages	13
2.7	Principal growth stages of flower and fruit development	14
2.8	Phenological cycle of sweet cherry	15
2.9	Double fruit due to high temperature in summer	21
2.10	Genes known to be involved in the pathway from vernalisation to flowering	30
2.11	Possible histone modifications	32
2.12	Spatial arrangement of chromatin modifications important for gene expression	33
2.13	Vernalisation-mediated changes in FLC chromatin	35
2.14	Four predominant chromatin states (CS)	37
2.15	ABA pathway involved in flowering repression through the activation of <i>FLC</i>	41
2.16	GA pathway involved in flowering time	42
2.17	Main changes in plants in response to cold temperature that lead to cold acclimation	48
4.1	Characteristics of varieties studied (part 2)	52
4.2	Dormancy release phases of sweet cherry flower buds according to the BBCH scale	53
4.3	Workflow for the test of chemicals on cut branches	55
4.4	Workflow for the test of Roullier products on trees	57
4.5	Application dates of Roullier products during winter 2017/2017 in different species	58
4.6	Workflow ChIP-seq and RNA-seq	63
4.7	Yeast Two Hybrid system	77
4.8	Trans activation/repression system	78
5.1	Dormancy status under environmental conditions and RNA-seq sampling dates	86
5.2	Chilling and heat accumulation for the three sweet cherry cultivars	87
5.3	Transcriptome accurately captures the dormancy state	88
5.4	Heatmap of differentially expressed genes in flower buds of three different sweet cherry cultivars during bud development	90
5.5	Specific expression patterns during bud development	91
5.6	Marker genes allow to distinguish each dormancy phase	92
5.7	Marker genes infer the sweet cherry developmental phase	93
S5.1	RNA-seq harvest time points	99
S5.2	Heatmap of genes for A) 'Cristobalina', B) 'Garnet' and C) 'Regina'	100
S5.3	RNA-seq metrics from the three sweet cherry cultivars studied	101
S5.4	Transcriptome accurately captures the dormancy state for the three different sweet cherry cultivars	101
S5.5	Heatmap of differentially expressed genes in flower buds of (A) 'Cristobalina' and (B) 'Garnet' sweet cherry cultivars during bud development	102
6.1	Conserved domains along PavDAM6, 5 and 4 protein sequences in sweet cherry	106
6.2	Transcriptional dynamics of <i>PavDAM</i> and <i>FT</i> genes in the flower buds of three sweet cherry cultivars during bud development	107

6.3	Protein-protein interactions between PavDAMs and FT	109
6.4	PavDAM6 is an activator transcription factor	109
6.5	Gene ontology (GO) enrichment for PavDAM5 targeted genes	110
6.6	PavDAM5 <i>in vitro</i> ChIP-seq profile	111
6.7	Proposed model of PavDAM interactions and their role in dormancy regulation in sweet cherry	115
S6.1	PavDAM5 <i>in vitro</i> ChIP-seq profile	116
7.1	Regulation of the Arabidopsis life cycle by the (A) PRC2 complexes and (B) PRC1 complexes	118
7.2	Dormancy status under environmental conditions	120
7.3	Heat map of differentially expressed genes in October, December and January in 'Burlat' flower buds 2014/2015	122
7.4	Histone 3 (H3) enrichment during dormancy	123
7.5	Tri-methylation of histone 3 at lysine 4 (H3K4me3) enrichment during dormancy	124
7.6	Tri-methylation of histone 3 at lysine 27 (H3K27me3) enrichment during dormancy	125
7.7	Transcriptional dynamics of genes associated with H3K4me [*] deposition in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016	126
7.8	Transcriptional dynamics of genes associated with H3K27m3 deposition in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016	129
7.9	Transcriptional dynamics of genes associated with H3K27m3 maintenance in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016	129
7.10	Effect of different Histone Deacetylase (HDAC) inhibitors on the sweet cherry dormancy status	131
7.11	Effect of different Histone Acetyltransferase (HAT) inhibitors on the sweet cherry dormancy status	131
7.12	Transcriptional dynamics of genes associated with histone acetylation in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016	132
S7.1	Histone 3 (H3) enrichment during dormancy replicate 2	137
S7.2	Tri-methylation of histone 3 at lysine 4 (H3K4me3) enrichment during dormancy replicate 2	138
S7.3	Tri-methylation of histone 3 at lysine 27 (H3K27me3) enrichment during dormancy replicate 2	139
S7.4	(A) Gene length per cluster and (B) H3K4me3 ChIP profile of one gene present in the cluster orange	140
S7.5	Gene ontology (GO) enrichment for the cluster 2 (orange) for 'Burlat' sweet cherry cultivar in 2014/2015	140
8.1	Biosynthesis and catabolism pathway for (A) ABA and (B) GAs	145
8.2	Effect of different GAs, ABA and their inhibitor on the sweet cherry dormancy status	149
8.3	Dormancy status under environmental conditions	150
8.4	Levels of endogenous bioactive GA4 and GA7, and precursors GA9 and GA19 in the flower buds of two sweet cherry cultivars during bud development	151
8.5	Transcriptional dynamics of genes associated with GA pathway in the flower buds of two sweet cherry cultivars during bud development	152
8.6	Levels of endogenous bioactive ABA and ABA conjugates in the flower buds of two sweet cherry cultivars during bud development	154
8.7	Transcriptional dynamics of genes associated with ABA pathway in the flower buds of two sweet cherry cultivars during bud development	156
8.8	ABA modelling	156
S8.1	Transcriptional dynamics of genes associated with ABA pathway in the flower buds of two sweet cherry cultivars during bud development	165
S8.2	Transcriptional dynamics of genes involved in ABA synthesis and	166

	degradation for the sweet cherry cultivar ‘Garnet’	
9.1	Effect of OKEN on the sweet cherry dormancy status during four years of experiments	170
9.2	Effect of OKEN on the sweet cherry dormancy status in 2017 in field	171
9.3	Effect of OKEN on budbreak and flowering time for a late cultivar ‘Fertard’ in field (2017)	171
9.4	Schedule of treatments for field experiment in 2018	172
9.5	Effect of OKEN on the sweet cherry dormancy status of an early cultivar ‘Folfer’ (2018)	173
9.6	Effect of OKEN on the sweet cherry dormancy status of a late cultivar ‘Fertard’ (2018)	174
9.7	Effect of OKEN and different formulation of OKEN on budbreak and flowering time on a late and an early sweet cherry cultivar and an apple cultivar in field (2018)	174
9.8	Transcriptional dynamics of genes associated with flowering and dormancy pathway in the flower buds of sweet cherry after OKEN treatments	175
9.9	Levels of endogenous phytohormones in the flower buds of sweet cherry after OKEN treatments	176
9.10	Ratio of reduced to oxidised glutathione (GSH/GSSG) in the flower buds of sweet cherry after OKEN treatments	177
9.11	Proposed mode of action for the OKEN-induced acceleration of budbreak in sweet cherry	179
10.1	Summary of key findings from this PhD thesis	186

List of Tables

Table number	Table title	Page number
2.1	Classification of genus <i>Prunus</i>	6
2.2	World production of sweet cherry (<i>Prunus avium</i> L.)	9
2.3	Most important countries for the exportation of cherries including sweet and sour cherry	9
2.4	Main cultivars planted in the top 5 producing countries and in France	16
2.5	Classical breeding program	17
2.6	Candidate genes located in the quantitative trait loci (QTLs) on linkage group 4 in sweet cherry (<i>Prunus avium</i> , L.)	26
4.1	Characteristics of cultivars studied (part 1)	51
4.2	List of chemicals used in this study and their concentrations	55
4.3	Application concentrations of Roullier products on ‘Fertard’ trees during winter 2016/2017	56
4.4	Application concentrations of Roullier products during winter 2017/2018	59
4.5	Bud harvest dates for ‘Burlat’ during winter 2014/2015 to perform RNA-seq and ChIP-seq	60
4.6	Bud harvest dates for different sweet cherry cultivars displaying different flowering date during winter 2015/2016 to perform RNA-seq	60

List of Supplemental Figures

Appendix number	Appendix title	Page number
1	Phenological growth stages of sweet cherry according to the BBCH scale	213
2	BBCH scales according to Meier <i>et al.</i> , 1994	214
3	Incompatibility groups (IGs) of selected sweet cherry cultivars	215
4	Sequencing data of the sweet cherry genome 'Stella'	216
5	Comparison of the two sweet cherry genomes 'Regina' and 'Satonishiki' sequences	216
6	A list of advantages of RNA-seq compared with other transcriptomic methods	216
7	Epigenetic drugs used in this study	217
8	Primers used in qPCR	217
9	Primers used in in vitro ChIP assay	218
10	CDS sequences of PavDAM4, PavDAM5, PavDAM6 and PavFT in <i>Prunus avium</i> L.	219
11	Primers used for the Yeast Two Hybrid assay	220
12	Primers used for the Trans activation/repression assay	220

Table of Contents

1. General introduction	1
2. Literature review	4
2.1 Introducing cherry	4
2.1.1 Taxonomy	4
2.1.2 Origin and domestication of cherry	7
2.1.3 Agricultural value in the world and in France	8
2.1.4 Tree architecture and bud development	11
2.1.5 Flowering phenology	14
2.1.6 Cherry breeding	16
2.1.7 Key points to remember	18
2.2 Adaptation of sweet cherry to climate change	19
2.2.1 Global warming and its past impact on cherry production	19
2.2.2 Impact of climate change on cherry phenology and fruit development	20
2.2.3 Strategies to tackle fruit losses	21
2.2.4 The key points to remember	22
2.3 Genetic resources	22
2.3.1 <i>Prunus</i> Biological Resources Centre (BRC)	22
2.3.2 European Cooperative Programme for Crop Genetic Resources (ECPIGR) <i>Prunus</i> working group	23
2.4 Genomic resources	23
2.4.1 Genomes	23
2.4.2 Cherry transcriptome	24
2.4.3 Cherry linkage map and Quantitative Trait Locus (QTL)	25
2.4.4 Key points to remember	26
2.5 Dormancy in perennials	27
2.5.1 Definition	27
2.5.2 Chilling and heat requirements	27
2.5.3 Mechanisms regulating bud dormancy	28
2.5.3.1 Main molecular pathways	29
2.5.3.2 Epigenetic regulation of dormancy and flowering	32
2.5.3.3 Hormonal regulation of dormancy	38
2.5.3.4 Other important pathways involved in dormancy	44
2.5.4 Cold acclimation	46
2.5.5 Key points to remember	48
3. Objectives	49
4. Materials and methods	51
4.1 Plant materials and methods	51
4.1.1 Cultivars studied	51
4.1.2 Natural growth conditions (orchards)	52
4.1.3 Measurements of bud break and estimation of the dormancy release date	52
4.1.4 Epigenetic drugs and Roullier products studied	53
4.1.5 Application of products on cut branches in controlled conditions (growth chamber)	54
4.1.6 Application of Roullier products on trees in natural conditions (orchards)	56
4.1.7 Material sampling	59
4.2 Molecular biology procedures	61
4.2.1 qRT-PCR reactions	61
4.2.2 ChIP-seq and RNA-seq	62
4.2.3 <i>In vitro</i> ChIP	72
4.2.4 Yeast-two-hybrid assays	76

4.2.5 Trans activation/repression assay	77
4.2.6 Phytohormone quantification	78
4.2.7 Sulphur metabolite quantification	79
5. Cherry bud developmental phases can be inferred from their transcriptional profiles	82
5.1 Introduction	82
5.2 Material and Methods	84
5.3 Results	88
5.3.1 Substantial differences in the dormancy release date and chill requirements between the three cherry cultivars	88
5.3.2 Developmental stages of flower buds are characterized by the differentially expressed genes	89
5.3.3 Expression patterns are associated with developmental stages	89
5.3.4 Phase-dependent transcriptional activation and repression are associated with specific pathways	92
5.3.5 A set of marker genes can define the bud dormancy state	92
5.4 Discussion	94
5.4.1 Physiological states of the buds are associated with transcriptomic changes	94
5.4.2 New insights about dormancy phases between different cultivars	95
5.4.3 Determination of bud developmental phases using a small gene subset: a decision-making tool for growers and researchers	96
5.5 Conclusion and on-going analyses	98
6. New insights about <i>DORMANCY ASSOCIATED MADS-BOX (DAM)</i> genes in the regulation of dormancy in sweet cherry (<i>Prunus avium</i> L.) floral buds	103
6.1 Introduction	103
6.2 Material and Methods	104
6.3 Results	106
6.3.1 DAM proteins sequences display domain/motif conservations	106
6.3.2 Waves of transcriptional activation and repression of <i>PavDAM</i> genes are observed during dormancy phases	107
6.3.3 Negative correlation between <i>PavFT</i> and <i>PavDAM</i> gene expressions during dormancy	108
6.3.4 Physical interaction of <i>PavDAM</i> proteins	108
6.3.5 <i>PavDAM6</i> acts as a transcriptional activator	108
6.3.6 Preliminary results of <i>PavDAM5</i> in vitro ChIP-seq suggest a targeting of abiotic stimulus response genes	110
6.4 Discussion	111
6.4.1 The key role of DAM genes in the regulation of dormancy phases	111
6.4.2 <i>PavDAM6</i> : transcriptional activator or repressor?	112
6.4.3 <i>PavDAM5</i> , a potential activator of cold response genes	114
6.5 Conclusion	115
7. H3K4me3 and H3K27me3 modifications are associated with dormancy progression in sweet cherry (<i>Prunus avium</i> L.) flower buds	117
7.1 Introduction	117
7.2 Material and Methods	119
7.3 Results	122
7.3.1 Identification of two dormancy phases in cherry flower buds: endodormancy and ecodormancy	122
7.3.2 Waves of transcriptional activation are observed during endodormancy and ecodormancy	126

7.3.3	Global changes in H3K4me3 and H3K27me3 during dormancy	127
7.3.4	Expression of genes involved in H3K4me [*] and Polycomb Repressive Complex 1 and 2 (PRC1; PRC2) depositions have distinct patterns during sweet cherry bud dormancy	127
7.3.5	Epigenetic drugs involved in histone acetylation/ deacetylation impact dormancy release	130
7.4	Discussion	132
7.4.1	The dormancy cycle does not involve global changes in histone acetylation but a fine balance between H3K4me3 and H3K27me3 might impact gene expression	132
7.4.2	A fine regulation of PRC1-2 seems to be implicated in flower transition and flower formation	134
7.5	Conclusion	135
8.	Hormonal balance finely tunes dormancy status in sweet cherry flower buds	141
8.1	Introduction	142
8.2	Material and Methods	145
8.3	Results	148
8.3.1	Exogenous GA application accelerates bud dormancy release	148
8.3.2	GA content changes during flower bud dormancy progression ...	149
8.3.3	Expression of GA pathway-related genes have distinct patterns during sweet cherry bud dormancy	151
8.3.4	ABA levels rise at the onset of dormancy	153
8.3.5	Analysis of differentially expressed genes involved in ABA pathway	155
8.3.6	Modelling suggests ABA levels control onset and duration of dormancy	157
8.4	Discussion	158
8.4.1	Sweet cherry specific GA signalling during bud dormancy	158
8.4.2	Three dormancy phases as defined by gibberellin signalling	158
8.4.3	GA regulation by deactivation during dormancy	159
8.4.4	Dormancy depth is correlated with endogenous ABA content	160
8.4.5	A contrasted balance between ABA synthesis and conjugation may explain the differences between early and late cultivars	161
8.4.6	Towards new phenology approaches based on molecular mechanisms	163
9.	OKEN: a new product impacting bud break in sweet cherry (<i>Prunus avium</i> L.)	167
9.1	Introduction	167
9.2	Material and Methods	168
9.3	Results	168
9.3.1	OKEN stimulates bud break and presents no toxicity for buds	168
9.3.2	Two applications of OKEN on sweet cherry trees increases bud break	170
9.3.3	OKEN impact the expression of dormancy-related genes and bud metabolism	174
9.4	Discussion	177
9.4.1	Similar physiological responses between dormancy-breaking reagents Dormex® and OKEN	177
9.4.2	Contrary to Dormex®, OKEN presents no risk for buds when applied at the wrong time-interval	179
9.5	Conclusion	180
10.	General discussion	181
	References	187
	Appendix	213

1. General introduction

The global population growth is exponential but resources are limited. Moreover global warming negatively impacts the agricultural production thereby exacerbating the food crisis. It was estimated in 2015 that 795 millions people suffer from malnutrition or starvation in the world (FAO, 2015). As a comparison, this is one hundred times more than people affected by cancer. In this context, it becomes urgent to adapt the agricultural practices to reduce the negative effects of climate change on world food production. An increase of global temperature of 2°C to 4°C is expected in 2050 (IPCC, 2013; [Figure 1.1](#)) and Battisti and Naylor (2009) predicted that an increase of 1°C may result in a decrease of 16% of crop production without accounting for associated water shortages ([Figure 1.2](#)). Indeed, it has been seen that during the European heat wave of 2003, crop yields dropped by about 30% (Ciais *et al.*, 2005).

An increase of temperature dramatically influences developmental processes such as flowering, which would be the most impacted for fruit trees (Wolfe *et al.*, 2005; Lu *et al.*, 2006). Indeed, in the current context of climate change, flowering time and winter dormancy in perennial species are affected in a complex way by increasing winter and spring temperatures (Menzel, 2000; Chmielewski and Rötzer, 2001), potentially resulting in lower fruit production. These phenomena may cause losses of billions of euros in the fruit industry (Snyder and Melo-Abreu, 2005). This is particularly true for sweet cherry trees (*Prunus avium* L.), which are highly sensitive to temperature variations.

The economic and social challenge is to develop new agronomic practices adapted to climate change in order to maintain the actual fruit production. In France, the fruit sector generated a turnover of three billions euros in 2010 (Agreste N°16, 2013) but in 1998, the French cherry production was divided by two due to climatic accidents (2.8 T/ha instead of 5 T/ha on average; FAOSTAT, 2014). This is why it becomes urgent to find new solutions to alleviate the effects of climate change. Three main options can be considered to maintain the fruit production: 1) move the production areas according to climatic predictions, 2) create new cultivars adapted to climate change and 3) apply reagents that act directly on the phenology cycle, to delay or accelerate dormancy release for example.

The first option is less economically feasible because the creation of new areas will be a large economic loss for the actual growers and for the regions. The second option seems to

be realistic but requires a better understanding of the molecular events regulating phenology and its control by temperature. Breeding strategies to obtain new varieties adapted to climate change are slow and will only be effective in up to twenty years. Unfortunately, the effects of global climate change are already perceived in different countries. Another solution with faster impact is to develop new products impacting the dormancy and flowering processes.

Genetic and genomic approaches have recently led to some progress in understanding the mechanisms underlying dormancy control in deciduous trees (Arora *et al.*, 1997; Welling *et al.*, 2004; Rohde and Bhalerao, 2007; Allona *et al.*, 2008; Horvath *et al.*, 2010; Jiménez *et al.*, 2010a; Tanino *et al.*, 2010; Ueno *et al.*, 2013), including epigenetic and molecular mechanisms (Meijón *et al.*, 2009; Santamaría *et al.*, 2011; Leida *et al.*, 2012a; Ríos *et al.*, 2014; De la Fuente *et al.*, 2015). The “Adaptation du Cerisier au Changement Climatique” group (INRA, UMR 1332, A3C) is a leader in the study of sweet cherry in the context of climate change. Among the projects led by the group, any possible network involved in dormancy and flowering are investigated to improve breeding strategies in sweet cherry, in collaboration with the team of Philip A. Wigge (Sainsbury Laboratory, University of Cambridge, UK). In addition, the A3C group works in partnership with the Roullier Group, an agrotech company, which develops environmental friendly innovative products, in order to test reagents affecting the dormancy cycle.

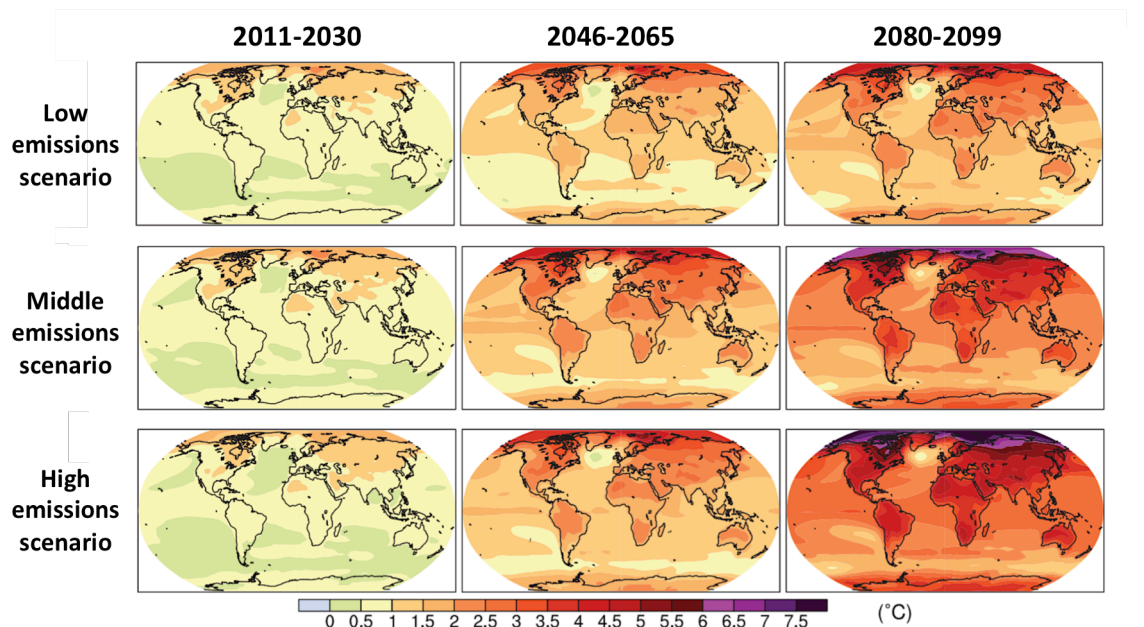


Figure 1.1 | Global warming projection

Models projection of the geographical pattern of annual average surface air temperature changes at three time periods (relative to the average temperatures for the period 1961–1990) for three different scenarios of greenhouse gas emissions (Meehl *et al.*, 2007).

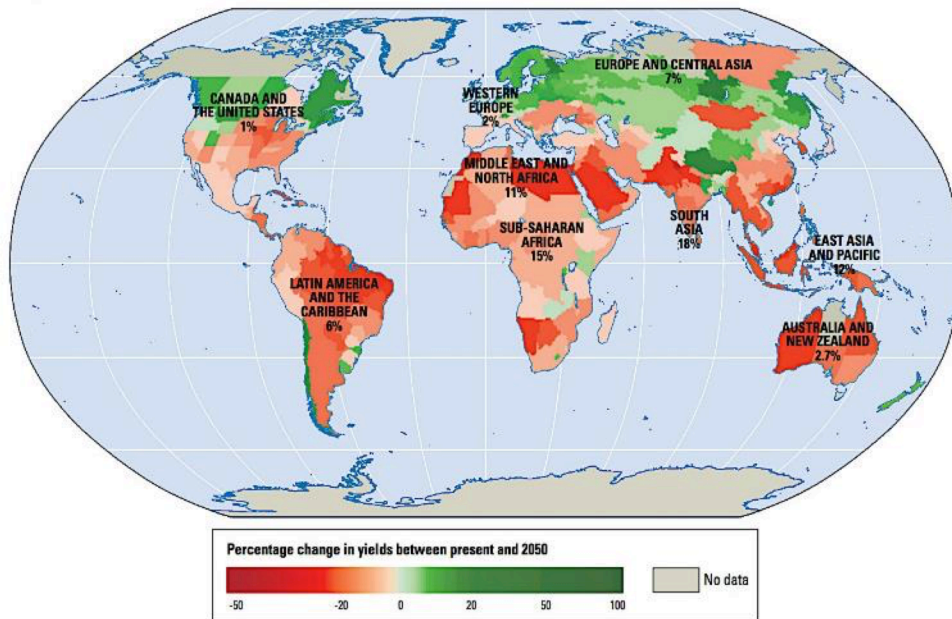


Figure 1.2 | Climate change will depress agricultural yields in most countries in 2050

Projected percentage change in yields of 11 major crops (wheat, rice, maize, millet, field pea, sugar beet, sweet potato, soybean, groundnut, sunflower, and rapeseed) from 2046 to 2055, compared with 1996–2005. The yield-change values are the mean of three emission scenarios across five global climate models, assuming no CO₂ fertilization (Müller *et al.*, 2009).

2. Literature review

2.1 Introducing cherry

2.1.1 Taxonomy

Cherries are members of the **Rosaceae family** and **Spiraeoideae subfamily** (Potter *et al.*, 2007). The *Rosaceae* family is composed of more than 90 genus and 3,000 species, with an important agronomic value (e.g. apple, peach, pear, almond, apricot, plum, strawberry, raspberry; **Figure 2.1a**). This family was defined by the observation of flowers in which the floral organs are organised in multiples of 5. According to Potter *et al.* (2007), the *Rosaceae* family is divided in three subfamilies: *Rosoideae*, *Dryadoideae* and *Spiraeoideae*. In the *Rosoideae* subfamily, genus *Fragaria*, *Rosa*, *Potentilla*, and *Rubus* are the most represented (**Figure 2.1a**) while in the *Spiraeoideae* subfamily, genus *Malus*, *Pyrus* and *Prunus* are the most represented. Cherry belongs to the genus *Prunus* (**Figure 2.1a**) and the tribe **Amygdaleae** (**Figure 2.1b**), which is composed of more than 200 crop species, members of the stone fruits, including peach/nectarine (*Prunus persica* (L.) Batsch), apricot (*Prunus armeniaca* L.), almond (*Prunus amygdalus* Batsch) and plum (*Prunus domestica* L. and *Prunus salicina* Lindl.) (**Figure 2.1c**). Cherries are further placed within two subgenera (*Cerasus* Pers. and *Padus* (Moench) Koehne) (Rehder, 1974; lezzoni *et al.*, 2017; **Table 2.1.1**). The subgenera *Cerasus* Pers. regroups *Eucerasus* (*P. avium* L., *P. cerasus* L.), *Pseudocerasus* (*P. serrulata*, *P. incise*), *Microserasus* (*P. japonica*, *P. besseyi*) and *Mahaleb* (*P. mahaleb*) (**Table 2.1**). Some of them are cultivated for their fruits (*P. avium* L., *P. cerasus* L., *Prunus x gonduinii* Rehd) or used as rootstock (*P. mahaleb*).

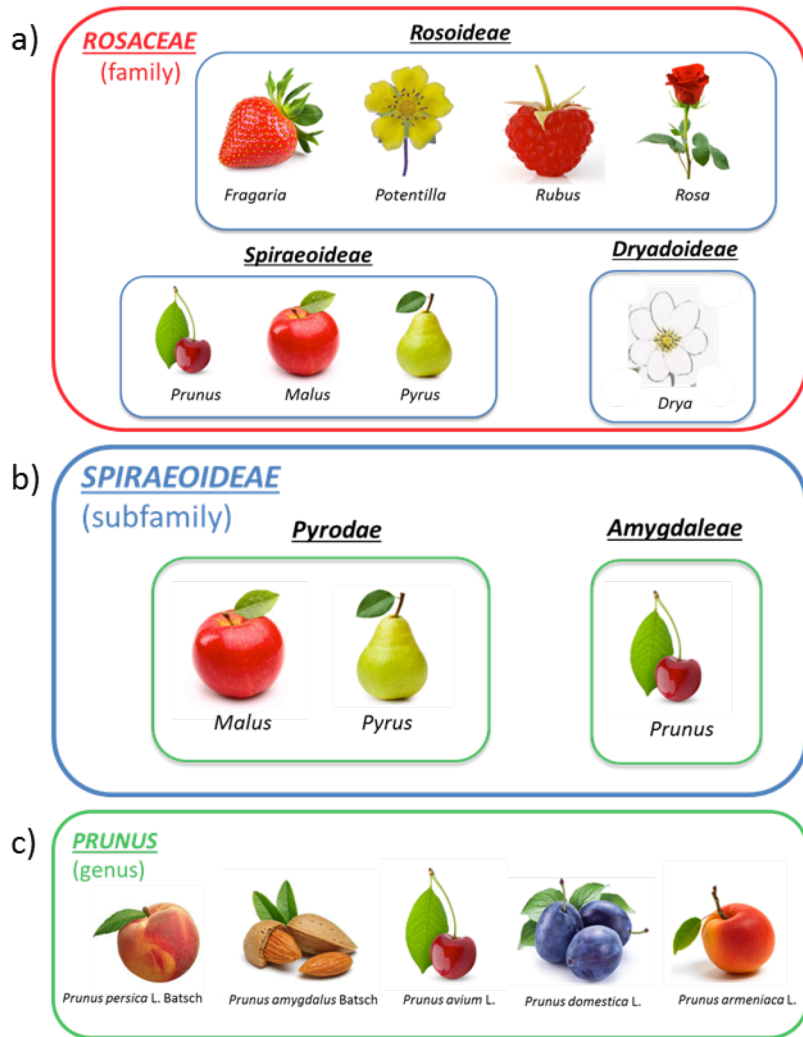


Figure 2.1 | Cherry taxonomy

According to Potter *et al.* (2007) and Rehder (1974)

a) *Rosaceae* family is divided in three subfamilies: *Rosoideae*, *Dryadoideae* and *Spiraeoideae*.

b) Within the *Spiraeoideae* subfamily, cherry is in the tribe *Amygdaleae*.

c) And in the genus *Prunus*.

Subgenus	Sections	Species	Distribution
CERASUS Pers.	Microcerasus ³ Webb	<i>P. besseyi</i> Bailey	Canada, U.S.A.
		<i>P. glandulosa</i> Thumb.	China, Japan
		<i>P. humilis</i> Bge	N. China
		<i>P. incana</i> (Pall.)	S.E. Europe, W. Asia
		<i>P. jacquemontii</i> Hook.	N.W. Himalayas
		<i>P. japonica</i> Thunb.	C. China, E. Asia
		<i>P. microcarpa</i> C.A. Mey	Asia Minor
		<i>P. prostrata</i> Labill.	Medit, W. Asia
		<i>P. pumila</i> L.	U.S.A.
		<i>P. tomentosa</i> Thunb.	N. & W. China, Japan, and Himalayas
	Pseudocerasus Koehne	<i>P. campanulata</i> Maxim	S. Japan, Formosa
		<i>P. cerasoides</i> D. Don	Himalayas
		<i>P. incisa</i> Thumb.	Japan
		<i>P. kurilensis</i> (Miyabe) Wils.	Japan
		<i>P. nipponica</i> Matsum	Japan
		<i>P. sargentii</i> Rehd.	Japan
		<i>P. serrulata</i> Lindl.	Japan, China, Korea
		<i>P. sieboldii</i> (Carr.)	Japan
		<i>P. subhirtella</i> Miq. ¹	Japan
		<i>P. yedoensis</i> Matsum	Japan
	Lobopatalum Koehne	<i>P. cantabrigiensis</i> Stapf.	China
		<i>P. involucrata</i> Koehne	C. China
		<i>P. pseudocerasus</i> Lindl.	N. China
		<i>P. dielsiana</i>	China
	Cerasus Koehne	<i>P. avium</i> L.	<u>Europe, W. Asia, Caucasus</u>
		<i>P. cerasus</i> L.	W. Asia, S.E. Europe
		<i>P. fruticosa</i> Pall.	C. & E. Europe, Siberia
		<i>P. canescens</i> Bois ²	C. & W. China
	Mahaleb Focke	<i>P. emarginata</i> (Hook.) Walp.	U.S.A.
		<i>P. mahaleb</i> L.	Europe, W. Asia
		<i>P. pennsylvanica</i> L.	Canada, U.S.A.
		<i>P. prunifolia</i> (Greene) Shafer	Canada, U.S.A.
	Phyllocerasus Koehne	<i>P. pilosiuscula</i> Koehne	C. & W. China
		Phyllomahaleb Koehne	<i>P. maximowiczii</i> Rupr.
PADUS (Moench) Koehne		<i>P. alabamensis</i> Mohr.	U.S.A.
		<i>P. buergeriana</i> Miq.	Japan, Korea
		<i>P. grayana</i> Maxim.	Japan
		<i>P. maackii</i> Rupr.	Manchuria, Korea
		<i>P. padus</i> L.	Europe, N. Asia, Korea, Japan
		<i>P. serotina</i> Ehrh.	Canada, U.S.A.
		<i>P. ssiori</i> F. Schmidt	N.E. Asia, Japan
<i>P. virens</i> (Woot. & Standl.)	U.S.A.		
<i>P. virginiana</i> L.	Canada, U.S.A.		

¹*P. lannesiana*, listed as a species used in rootstock breeding, is considered to be a subspecies of *P. serrulata*.

²According to its breeding behavior, it is proposed that *P. canescens* belongs to the *Cerasus* section (Schmidt, 1973).

³*Microcerasus* has been recently included in the *Prunus* subgenus using phylogenetic analysis on twelve chloroplast regions and three nuclear genes (Shi et al., 2013).

Table 2.1 | Classification of genus *Prunus*

Prunus avium L. is a member of *Cerasus* Pers. subgenus and more precisely of *Cerasus* Koehne (Iezzoni et al., 2017) according to Rehder (1974).

2.1.2 Origin and domestication of cherry

A large spectre of cherry species exists in the world with different morphologies and either producing edible fruits or not. Three species have been selected for fruit production: ***Prunus cerasus* L. (sour cherry)**, ***Prunus avium* L. (sweet cherry)** and ***Prunus x gondouinii* (duke cherry)**.

Prunus avium have originated around the Caspian and Black Sea, with the Caucasus area considered being a major centre of genetic diversity (Hedrick *et al.*, 1915; Webster, 1996; Dirlewanger *et al.*, 2009). From this region, *Prunus avium* propagation in Europe was mainly managed by birds that spread the seeds (Figure 2.2; Dirlewanger *et al.*, 2009; Zohary *et al.*, 2012). Fruit tree domestication appeared after seed plant domestication. Cereals, legumes, oils seeds were the first cultivated plants in 8,000 B.C. In the Early Bronze Age (first half of the fifth millennium B.C.), olive, grape vine, fig and date palm seem to have been the first principal fruit crops domesticated (Zohary *et al.*, 2012). The potential reason of this difference in domestication time is due to the quick production of seeds by annual plants. Before fruit tree domestication, humans harvested wild berries in the forests (Chauvet, 1999).

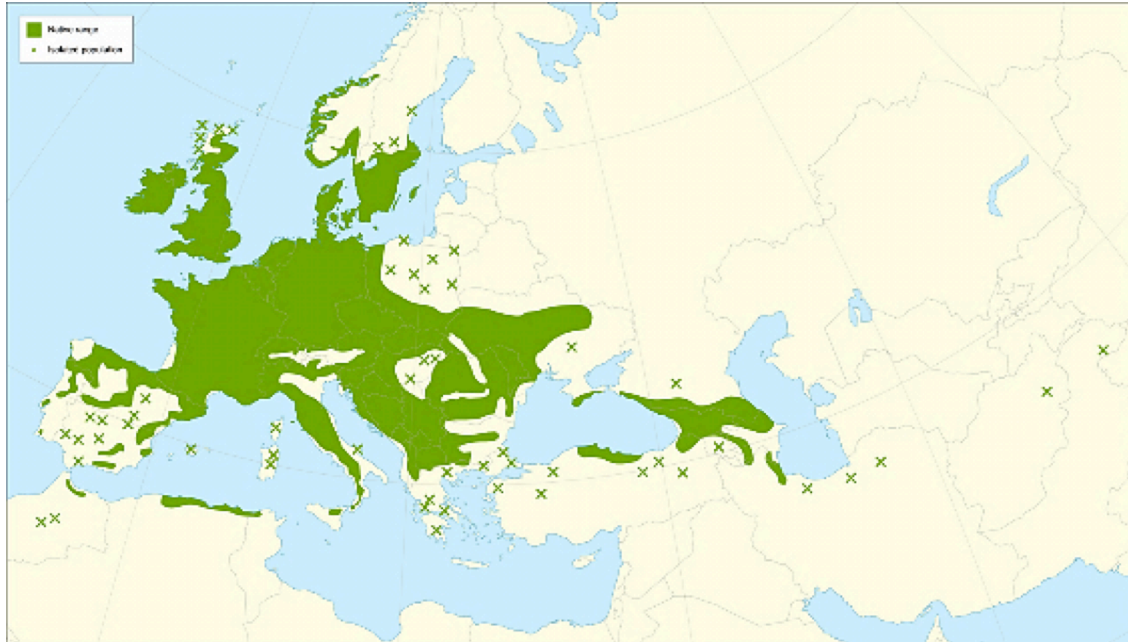


Figure 2.2 | Distribution map of wild cherry (*Prunus avium* L.)

The map was created combining numerous and heterogeneous data collected from 20th Century atlas monographs and from more recent national to regional atlases, occurrence geodatabases and scientific literature (Caudullo *et al.*, 2017.).

Apple, pear, plum, and cherry seem to have been added much later, since definite signs of their domestication appear only in the first millennium B.C. Their cultivation is almost entirely based on grafting, so their extensive domestication has happened only after the introduction of this sophisticated method of vegetative propagation (Zohary *et al.*, 2012). Sour cherry seems to have been introduced by Slavic people from western Asia to eastern and south-east Europe and from there to Middle Europe in the time of the Migration period (6th to 8th centuries; lezzoni *et al.*, 2017). The first sweet cherry grafting may have appeared in the Ancient Greece, and Romans then used this method and spread it during their territorial expansions (Chauvet, 1999). Studies on sweet cherry stones from an archaeological site (Hotel-Dieu Tours, France) suggested that different cultivars were already cultivated in the 16th century (Burger *et al.*, 2011). Louis XIV (i.e. King of France in the 17th century), who loved cherries, encouraged the production of cherry varieties in France. Cherries were brought to America in the 19th century, and from the east coast they were taken by early pioneers to the American west (lezzoni *et al.*, 2017). Sweet cherry cultivation has advanced rapidly since the 1990s, with the development of intensive orchards systems. This revolution was initiated by the arrival of dwarfing or semi-dwarfing precocious rootstocks, which reduced tree height and allowed higher tree densities, earlier yields and improved labour efficiencies (Quero-Garcia *et al.*, 2017). Today, cherry trees are found in most temperate zones (Chauvet, 1999).

2.1.3 Agricultural value in the world and in France

The annual global sweet cherry production is about **2.2 million tons**, with a rising tendency (Bujdoso and Hrotkó, 2017; FAOSTAT, 2018; [Table 2.2](#)). The top ten producing countries are: Turkey, followed by the USA, Iran, Italy, Spain, Chile, Ukraine, Russian Federation, Romania and Poland ([Table 2.2](#)). Turkey produces almost 500,000 tons of cherries annually, representing approximately one quarter of the world production (Bujdoso and Hrotkó, 2017; FAOSTAT, 2018). During the period of 1980-2016, the sweet cherry production in Turkey, USA, Chile and China has drastically increased, while production in France, Germany, and Bulgaria markedly decreased (Bujdoso and Hrotkó, 2017; FAOSTAT, 2018; [Table 2.2](#)).

Country	1980	1990	2000	2010	2016
Turkey	96	143	230	417	599
USA	155	142	185	284	288
Iran	53	85	216	251	220
Italy	119	100	125	115	94
Spain	79	54	112	85	94
Chile	5	13	31	60	123
Ukraine	-	-	76	73	63
Russian Federation	-	-	85	66	46
Romania	40	40	43	42	73
Poland	25	9	35	41	53
China	-	-	8	28	37
France	112	82	66	44	34
Germany	71	80	44	30	29
Bulgaria	55	71	28	24	38
Australia	4	5	6	13	18
Japan	15	16	17	20	19

Table 2.2 | World production of sweet cherry (*Prunus avium* L.)

The most important countries for sweet cherry production (1000 t) (Bujdoso and Hrotkó, 2017 and FAOSTAT, 2018). -, data unavailable. The top ten producing countries are highlighted in red.

In terms of cherry exportation, 362,000 tons were exported in 2015/2016, the top three exporting countries being Chile, the USA and Turkey. For example, around 69,000 tons of Turkish cherries were exported in 2015/2016 (USDA-FAS, 2017; Table 2.3).

Country	2012/13	2013/14	2014/15	2015/16
USA	101	67	88	75
Chile	51	67	103	84
Turkey	57	54	50	69
European Union	30	43	39	48
Uzbekistan	5	17	17	5
Serbia	11	19	12	8
Azerbaijan	7	4	10	12
Canada	8	6	9	12
Belarus	1	3	7	38
Australia	3	3	4	6
Other	15	18	12	6
Total	288	301	351	362

Table 2.3 | Most important countries for the exportation of cherries including sweet and sour cherry

The USA, Chile and Turkey are the biggest exporting countries of sweet cherries (USDA-FAS, 2017)

In France, sweet cherry is the second most preferred fruit after strawberry and before melon (La Dépêche, 2014). However, the French sweet cherry production decreased during the period of 1980-2016 from 112,000 tons to 34,000 tons (Table 2.2; FAOSTAT, 2018) making cherry an expensive fruit. The most important growing areas are:

- **Provence-Alpes-Côte d'Azur (PACA)** with 47% of the national sweet cherry production in 2016
- **Auvergne-Rhône-Alpes** (23%)
- **Occitanie** (18%)

The combination of these regions represents 88% of the French national production in 2016 (Agreste Conjoncture_Cerise N°2017-100, 2017; Figure 2.3). The number of French growers is high (more than 4,000 in 2010) but 75% of the orchards have a small-cultivated surface (less than 2 hectares; Agreste Primeur N°277, 2012). Cherries are a high-risk fruit crop to grow susceptible to many damages (insects, plant diseases, winter frost, and fruit cracking) and require a large labour force to handpick the fruit, thus making prices higher. These constraints mentioned above might explain the decrease of production met over the last decades. Nonetheless, a few associations emerge to re-establish local varieties adapted to the climate. For example in the Basque Country at Itxassou (South West of France), the XAPATA association created in 1994 has led to the rehabilitation of local cherry varieties with success.

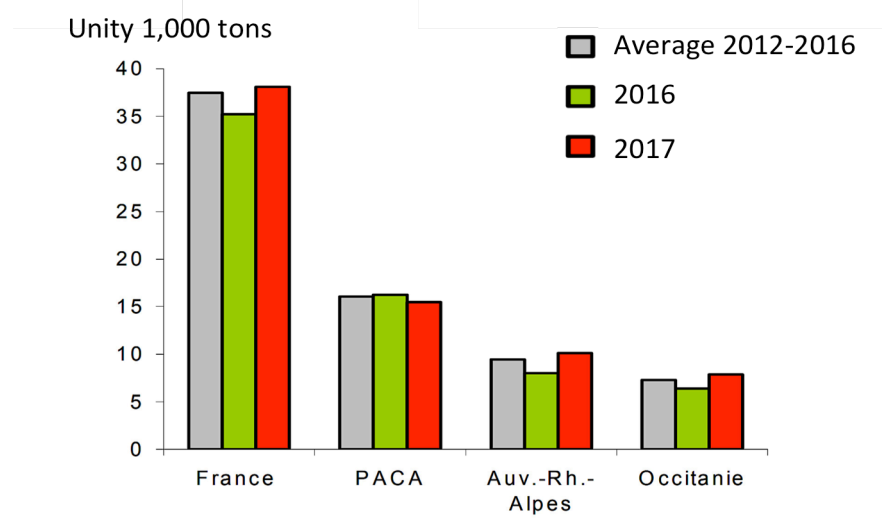


Figure 2.3 | French production of sweet cherry (*Prunus avium* L.) in 2016 and 2017 PACA (Provence-Alpes-Côte d'Azur), Auvergne-Rhône-Alpes and Occitanie are the most important growing area in France. The cherry production in 2017 appears to be a little bit higher than previous years (Agreste Conjoncture_Cerise N°2017-100, 2017).

More recently, a new threat has appeared for cherry production. *Drosophila suzukii*, a fruit fly native of Asia, has in recent years emerged as a global pest on soft-skinned fruits, and more

particularly for cherries (Maderspacher and Stensmyr, 2017), with yield losses up to 80% in heavily infested areas. *D. suzukii* was first spotted in France in 2009 and has been spreading in the territory without any mitigation solution so far.

2.1.4 Tree architecture and bud development

In the cherry tree, growth and branching are strictly monopodial (i.e., growth upward from a single point) at all stages of growth. The framework of the tree is composed of the trunk, with long and upright side-branches that usually develop with a strong acrotony (i.e. the increasing size of comparable lateral shoots toward the apex of the main shoot; Powell, 1995), forming tiers of branches just below the annual growth arrest of the bearing shoot (Costes *et al.*, 2014). Two life steps are identified:

- **The juvenile phase** when the tree does not produce gametes and has only vegetative buds. Only long vegetative branches are grown that build the tree's architecture. This step lasts between 3 to 5 years.

- **The adult/mature phase** when gametes are produced annually. After transition to reproductive phase, trees bear long and short shoots, with a strong dimorphism between them. The first flower buds appear at the bottom of the new long branches (Figure 2.4). The long branches, two years old or more, bear short shoots, also called spurs. A spur is a group of two to five flower buds in umbels with a vegetative bud in the middle (Figure 2.5).

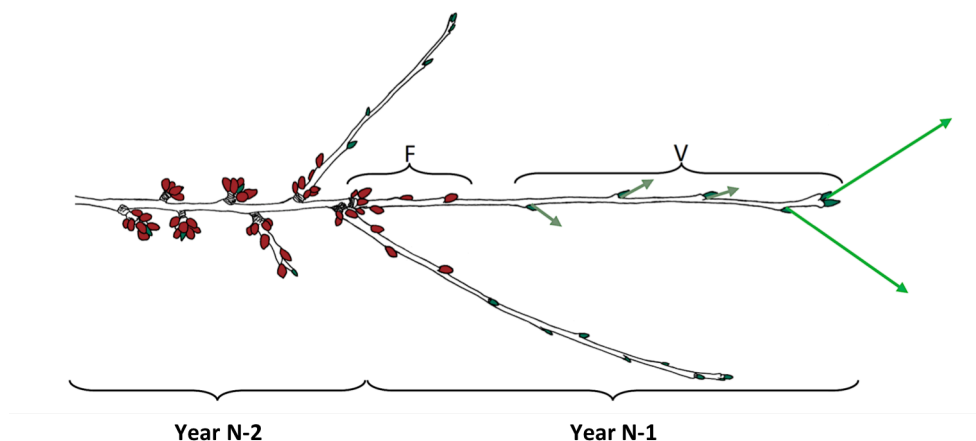


Figure 2.4 | Branching pattern along a cherry annual branch

The N-2 part of the branch is the neoformed part and N-1, the preformed part. The red buds are the floral buds, the green buds are the vegetative buds. The small green arrows indicate the future short branches and the longest arrows indicate the future long branches (from Bénédicte Wenden, personal realisation)

These flower buds will burst in the following year leaving bare wood after fruit harvest on the proximal part of long shoots, and on all nodes of the annual growth increment of the spurs

(Costes *et al.*, 2014). As a consequence, the typical branching pattern on the one-year-old branch consists in an initial basal zone with single axillary buds containing inflorescences directly, followed by a zone of spurs and a top tier of long lateral shoots (Costes *et al.*, 2014; [Figure 2.4](#)).

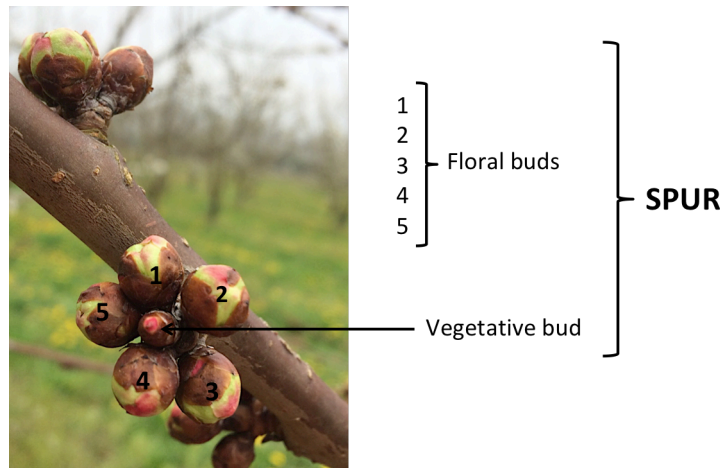


Figure 2.5 | Picture of a cherry spur

A cherry spur is composed of several floral buds in umbel with a vegetative bud in the middle.

Buds development occurs over two years: emergence close to the leaves from the year N, followed by organogenesis during summer then burst during the spring of year N+1.

From summer to fall, flower buds grow and specific floral tissues develop. The transition from a vegetative (dome shape) to a reproductive (establishment of flower primordia) meristem is marked by a shift in organogenetic activity in summer (Engin and Ünal, 2007). Floral organogenesis begin with the initiation of 5 sepals in mid-summer and pistil primordia are formed in a short period of time in late summer (Engin and Ünal, 2007). In late summer, as the photoperiod decreases, buds are covered by dark brown scales to protect sensitive tissues from cold, and flower primordia stop growing. After the winter rest period, floral buds resume active growth under favourable conditions at the end of winter. From this step to flowering in spring, each reproductive part of the flower emerge (pistil, sepal, anther, etc.). See the following [Figure 2.6](#) for the detailed explanations on the floral bud development (Fadón *et al.*, 2015). The pictures give a good overview of the bud development using the BBCH scale adapted to sweet cherry ([Appendix Figures 1 and 2](#); Meier *et al.*, 1994; Fadón *et al.*, 2015).

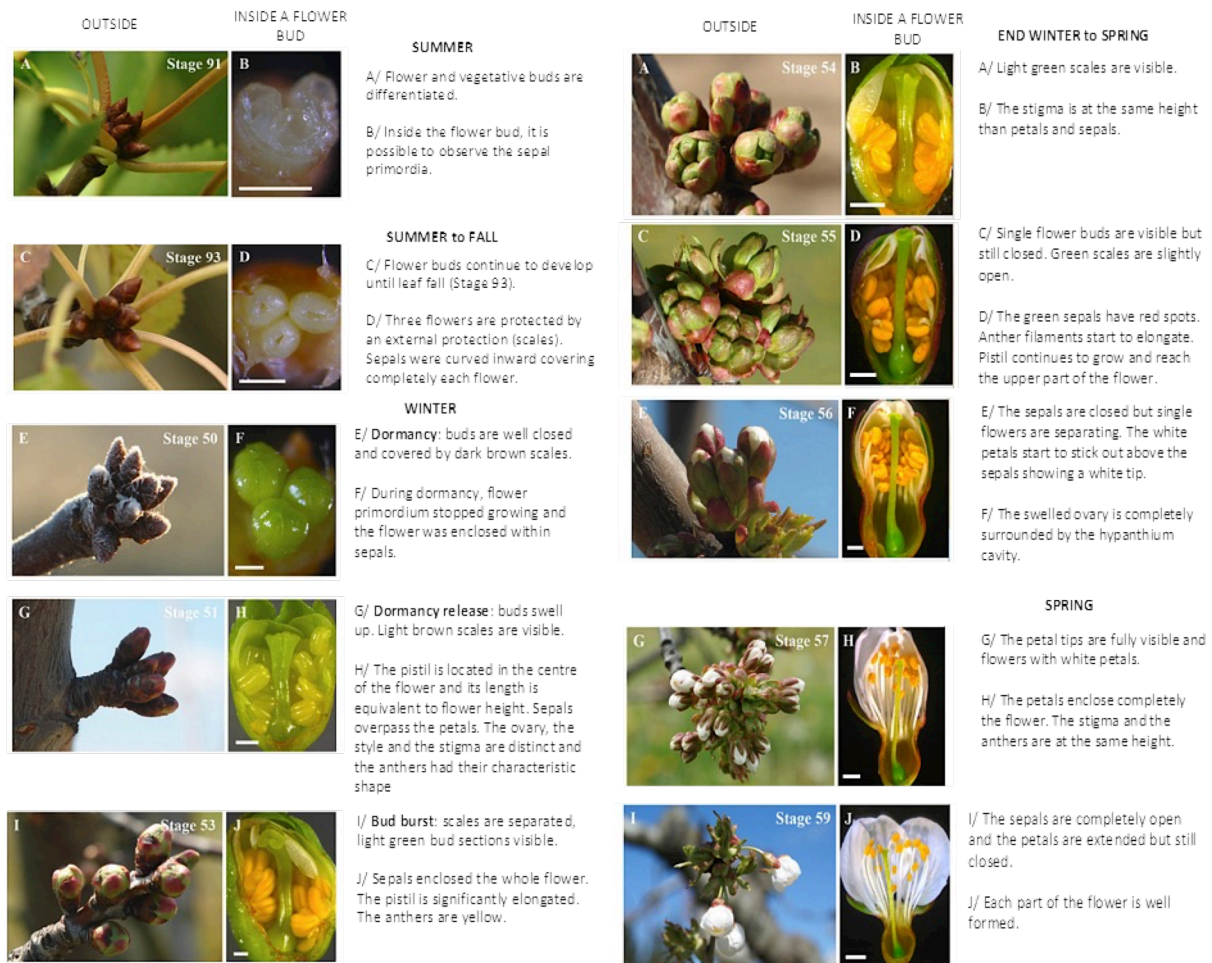


Figure 2.6 | Cherry flower development stages

Bud development of sweet cherry from summer (A to D) to winter (E to J), end of winter to spring (A to F), and spring (G to J) according to the extended BBCH scale. Scale bar = 0.2mm (Fadón *et al.*, 2015).

The cherry tree is a **cross-pollinated plant pollinated by bees and bumblebees** (*Apis* and *Bombus*). As several species in the *Prunus* genus, cherry is self-incompatible due to the presence of the S gene found in the linkage group six (Dirlewanger *et al.*, 2004). It is therefore necessary to know the S-genotype of each cultivar to create compatible cultivar combinations within an orchard. Cultivars with the same S-genotype are cross-incompatible and are grouped in the same incompatibility group (IG). In contrast, cultivars with different S-genotypes are cross-compatible and are therefore assigned to different IGs. Cultivars with unique S-genotypes, and thus compatible with all other cultivars of known S-genotype, are classified in group 0 comprising universal donors (Herrero *et al.*, 2017; [Appendix Table 3](#)). To avoid the use of pollinator trees, self-compatible cultivars are often preferred in cherry production. The predominant self-compatible mutation S_4' was spread in sweet cherry cultivars due to the common ancestry of the cultivar 'Stella', which derives from the radiation-induced mutant allele from J12420. Among self-compatible cultivars that do not carry the S_4'

allele, the very early cultivar ‘Cristobalina’ is of interest since its self-compatibility is not due to the S-locus (Iezzoni *et al.*, 2017). Following pollination, fruit set occurs and the ovary starts developing into the fruit.

2.1.5 Flowering phenology

The flowering and fruiting dates depend on several parameters such as the cultivar, the geographical distribution of the orchards, climatic conditions, type of rootstock, soil and agricultural practices.

Floral induction and initiation at the end of spring and summer mark the beginning of the flower bud cycle. Following organogenesis in the summer, leaves fall and cold acclimation are triggered as a result of decreasing photoperiod and temperatures (Mølmann *et al.*, 2005; Ruttink *et al.*, 2007; Tanino *et al.*, 2010). Then, buds cease their development and enter a state of dormancy from autumn to winter (Allona *et al.*, 2008). Following an increase in day length and temperature at the end of winter, buds resume their growth in spring, leading to flowering and fruit production (Allona *et al.*, 2008; Figure 2.7 and Figure 2.8). In temperate zones of the northern hemisphere, maturity for sweet cherry cultivars is reached between the end of April (in southern growing regions) and June-July (main season), while the picking season finishes in late August in Norway (Bujdosó and Hrotkó, 2017).

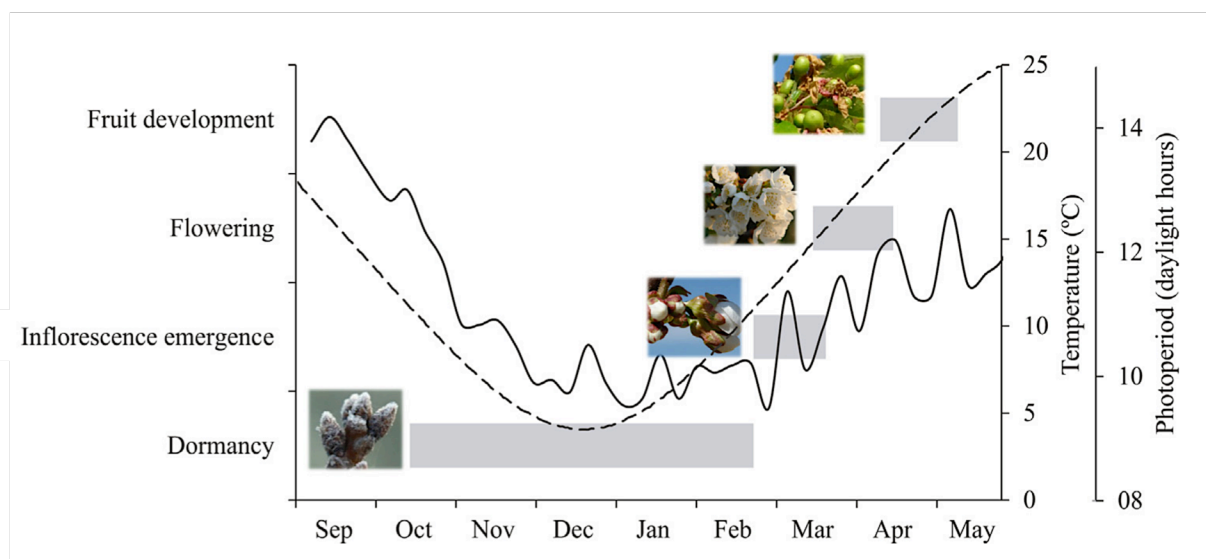


Figure 2.7 | Principal growth stages of flower and fruit development

Time elapsed in each stage (horizontal bars), weekly mean temperature (2012–2013) (continuous line) and photoperiod (dashed line) (Fadón *et al.*, 2015).

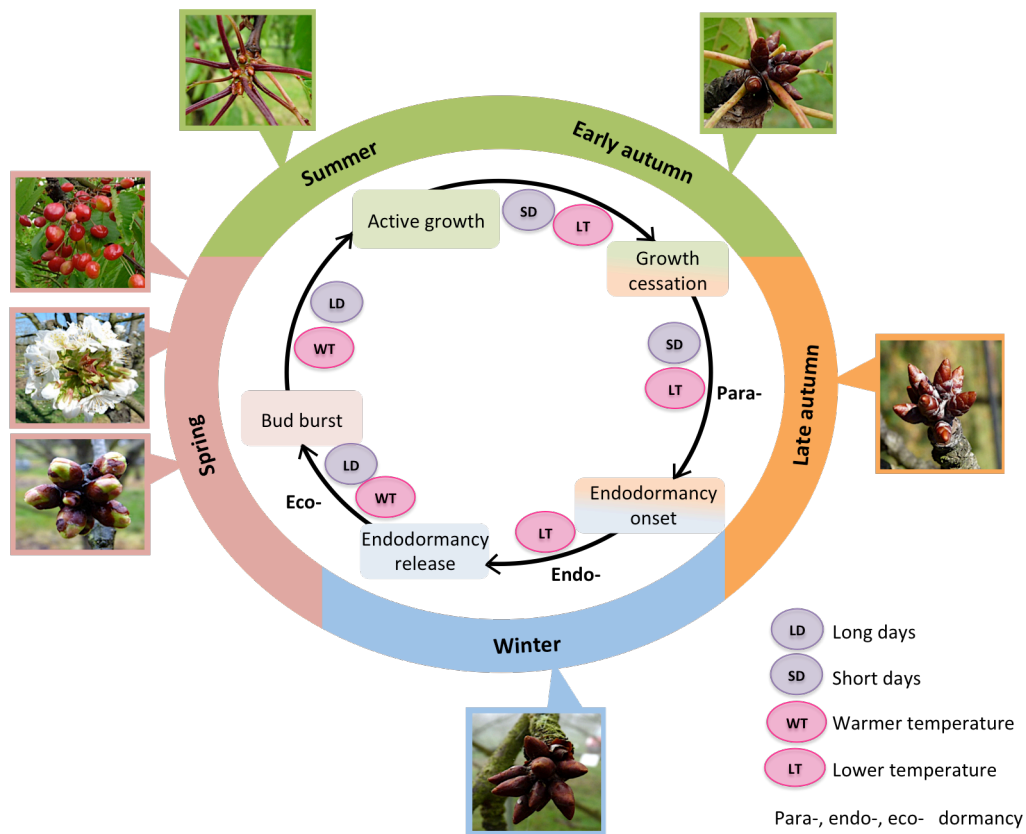


Figure 2.8 | Phenological cycle of sweet cherry

In perennial species, including sweet cherry trees, optimal flowering is highly dependent on the release of bud dormancy. Dormancy is a key strategy that allows trees to persist under low winter temperatures and short photoperiod (Faust *et al.*, 1997), enabling the successful reproduction of perennial fruit species including cherry (Zhu *et al.*, 2015), peach (Yamane *et al.*, 2011; Leida *et al.*, 2012a; De la Fuente *et al.*, 2015), pear (Saito *et al.*, 2015) and apple (Mimida *et al.*, 2015). Dormancy can be separated into three stages: **paradormancy**, **endodormancy** and **ecodormancy**, as defined by Lang *et al.*, (1987). (Figure 2.8), which are associated with chill requirements, i.e. cold accumulation necessary to release endodormancy, and heat requirements, related to warm temperatures necessary for the buds to burst in the spring. More details on chill/heat accumulation and dormancy progression will be presented in sections 2.5.2 (page 27). Once dormancy is released, buds may resume their growth and flower under the control of warmer temperatures and longer photoperiod during springtime (Figure 2.8).

Country	Main cultivars		
Turkey	'0900 Ziraat' 'Stark Gold' 'Regina'		
USA	California	Washington and Oregon	Michigan
	'Bing' 'Burlat' 'Brooks' 'Coral' 'Champagne' 'Chelan' 'Early Garnet™' 'Garnet' 'Rainier' 'Royal Rainier' 'Tulare'	'Bing' 'Chelan', 'Santina', 'Tieton' Early Robin™ 'Benton', 'Rainier', 'Attika' 'Kordia' 'Lapins'	'Emperor Francis' 'Gold' 'Napoléon' 'Sam' 'Ulster'
Iran	'Sorati Lavasan' 'Zarde Daneshkade' 'Shishei' 'Siahe Mashhad' 'Bing' 'Lambert' 'Van'		
Italy	'Burlat' 'Early Lory' 'Giorgia' 'Van' and 'Ferrovia'		
Spain	'Earlise®Rivedel'	'Santina'	
	'Burlat' 'Chelan' 'Prime Giant' 'Nimba' 'Pacific Red' 'Frisco' 'Crystal Champaign' 'New Star' 'Starking Hardy Giant' 'Lapins' 'Skeena'	'13S 3-13' '4-84' 'Van' 'Summit' 'Sunburst' 'Ambrunes' 'Lambert' 'SP-106' 'Sommerset' 'Sweetheart'	
France	'Burlat' 'Belge' 'Summit' 'Sweetheart' 'Napoléon' 'Folfer' 'Regina'		

Table 2.4 | Main cultivars planted in the top 5 producing countries and in France
According to Bujdosó and Hrotkó (2017).

2.1.6 Cherry breeding

Although cherries have been cultivated for more than 2000 years, cherry breeding is quite recent. Hedrick *et al.* (1915) described the beginning of sweet cherry breeding around the early 1800s, but even today, some modern cultivars are just a few generations away from early ancestors (Iezzoni *et al.*, 1990). Breeding programs were initiated in Geneva (1911; New York, USA), in Vineland (1915; Ontario, Canada) and in Summerland (1924; British Columbia, Canada) (Faust and Suranyi, 1997). European breeding programmes followed, such as the programme at John Innes in England that began in 1925 (Faust and Suranyi, 1997). Owing to these breeding programs, new cherry cultivars were created to improve fruit

yield (see Table 2.4 for the list of main cultivars planted in the top 5 producing countries and in France).

The breeding methods used are **clonal selection** and **selective reproduction** (trait selection). In the case of controlled breeding, the pollination is done manually or using pollinators, such as bumblebees, in a confined structure. As cherry has a long juvenile period, the introduction of quality traits in improved cultivars is a long process (approx. 24 years). Breeding new cherry cultivars is based on three main phases: 1) **varietal creation**, 2) **plant breeding** and 3) **varietal output** (Table 2.5).

Phase	Year	Step	Plant status	Period of time
Varietal creation	0	Parent breeding	Before flowering	End of Winter
		Hybridation	During flowering time	March-April
		Fruit harvest	Fruit maturity	June-July
		Embryo culture	Embryo dormancy	August to December
		Greenhouse growing	Seedlings	December to April
	1	Acclimation period	Plant	May to June
		Growth (tree nursery)	Young tree	June (n) to September (year n+1)
	2	Planting tree in the orchards	Young tree	Winter
Plant breeding	3 to 7	Juvenile period	Hybrid level	All year round
	7 to 9	First fruit harvest and selection	Hybrid level	June-July
	10	Grafting	Rootstock/Graft (hybrid x)	August to September
	11	Planting tree in the orchards	Level 1	Winter
	14 to 16	First fruit harvest and selection	Level 1	June-July
	17	Grafting	Rootstock/Graft (hybrid x)	August to September
	18	Planting tree in the orchards	Level 2	Winter
	21 to 23	First fruit harvest and selection	Level 2	June-July
Varietal output	24	New variety	Commercialisation level	

Table 2.5 | Classical breeding program (Beauvieux, 2017)

The main selection criteria for cherry breeding according to Quero-Garcia *et al.*, (2017) are:

- Yield precocity, productivity and, more recently, regularity of production.
- Fruit weight and firmness, tasting quality (balance between sweet and acid taste).
- Tolerance to fruit cracking.
- Self-fertility

In the medium to long term, new traits such as tolerance/resistance to pests and diseases (e.g. *Pseudomonas spp.* (responsible of bacterial canker), *Monilinia* (fungus responsible of blossom blight and brown rot on flowers and fruits, respectively)) or tolerance to biotic stresses (e.g., low chilling requirements, low susceptibility to double fruit formation) will be added to the trait selection list (Quero-Garcia *et al.*, 2017).

Over the past decades, a complementary approach based on **molecular markers** has been used to assist the selective breeding strategies. Early selection with molecular markers allows an accurate screening of seedlings several years before the characters can be evaluated in the field. This has also the advantage of allowing the accumulation of different resistance factors in a genotype of interest, or to shorten the number of generations needed to recover the genotype of the cultivated species after a cross with an exotic genotype or wild species (Dirlewanger *et al.*, 2004). Indeed, marker-assisted selection has recently been implemented for several key traits, such as self-incompatibility and fruit weight, and will presumably extend further to other traits as genomic research is tied to horticultural traits of interest.

2.1.7 Key points to remember

- The annual global sweet cherry production is about **2.2 million tons**.
- The top ten producing country leaders are: **Turkey**, followed by the **USA**, **Iran**, Italy, Spain, Chile, Ukraine, Russian Federation, Romania and Poland.
- In France, the sweet cherry production decreased during the period of 1980-2016 **from 112,000 tons to 34,000 tons**. Nonetheless, cherries ripen first among temperate tree fruits allowing a financial input in the early season.
- The cherry tree is an allogamous, **cross-pollinated plant, pollinated by bees**.
- In perennial species, including sweet cherry trees, optimal flowering is highly dependent on the release of bud dormancy. **Dormancy** is a key strategy that allows trees to persist under low winter temperature and short photoperiod enabling the successful reproduction of perennial fruit species.

- Dormancy can be separated into three stages: **paradormancy**, **endodormancy** and **ecodormancy**, associated with the response to temperature (chill and heat requirements).
- **Photoperiod** and **temperature** control each phenological phase during the bud life until the fruit stage.
- **Cherry breeding** allows the creation of new varieties with specific agronomic traits to improve cherry production.

2.2 Adaptation of sweet cherry to climate change

2.2.1 Global warming and its past impact on cherry production

With an estimated increase of global temperature between 2°C to 4°C in 2050 (IPCC, 2013; [Figure 1.1 bis](#)), global warming is expected to have considerable impacts on crop species including cherry. Additionally, Battisti and Naylor (2009) predict that even an increase of 1°C may result in a 16% decrease of crop production without accounting for associated water shortages. Climate change is also associated with a higher frequency of extreme events that vary across regions, including drought, hail, freezing temperature as well as heat waves and damaging levels of rain (Cubasch *et al.*, 2001). Increasing global temperature and its impact on crop production have already been reported. For example, during the European heat wave of 2003, crop yields dropped by about 30% (Ciais *et al.*, 2005). In 1998, the French cherry production was divided by two due to climatic accidents (2.8 T/ha instead of 5 T/ha on average; FAOSTAT, 2014).

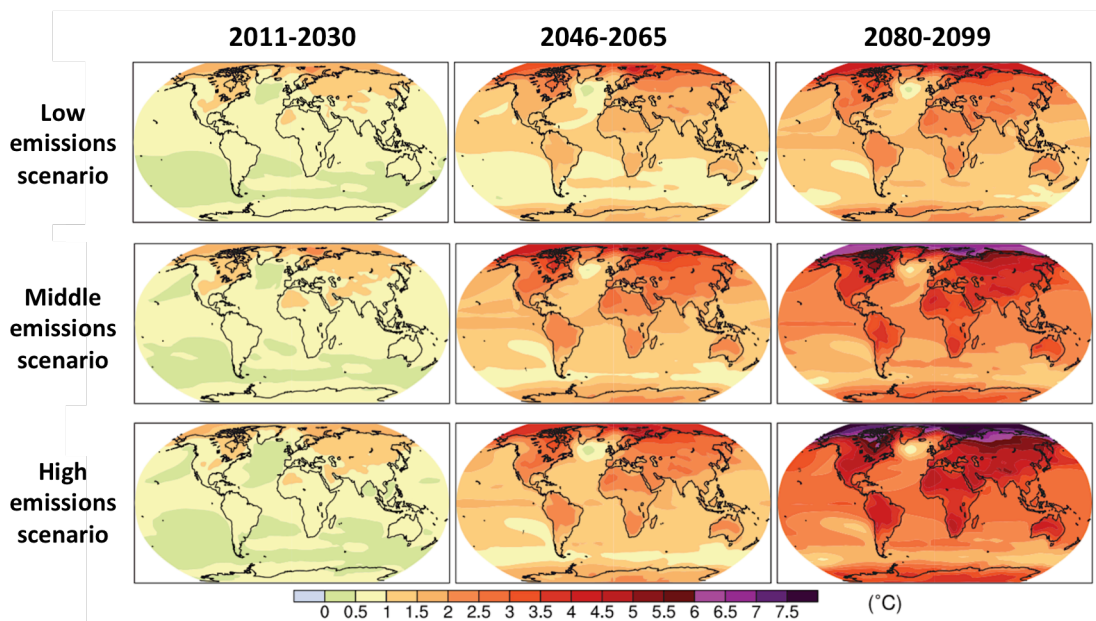


Figure 1.1 bis | Global warming projection

Models projection of the geographical pattern of annual average surface air temperature changes at three time periods (relative to the average temperatures for the period 1961–1990) for three different scenarios of greenhouse gas emissions (Meehl *et al.*, 2007).

In France, whatever the modelled climatic scenarios, mean temperature might increase between **0.6°C** (North of France) to **1.3°C** (South of France) by 2050 (Ouzeau *et al.*, 2014). These elevated temperatures might be associated with an **increase in the number of heat wave days** during summer (plus five to ten days mainly in South-East) and **less cold days in winter** (minus one to five days) (Ouzeau *et al.*, 2014), potentially associated with more drought phenomena during summer. In addition, an **increase of precipitations** is also expected in all seasons (between 0 to 0.45 mm/day) (Ouzeau *et al.*, 2014).

2.2.2 Impact of climate change on cherry phenology and fruit development

In this context of global warming, as flower buds growth and development are temperature-dependant, it is legitimate to question: how flowering can be fully achieved with milder winter temperature and warmer temperature in spring and summer?

Recent studies have highlighted important alteration in phenology due to the current global climatic changes. Especially, advances in spring phenology resulting from warming in most northern-hemisphere regions have been extensively documented (e.g. Badeck *et al.*, 2004; Menzel *et al.*, 2006; Fu *et al.*, 2015). This trend has also been observed with sweet cherry phenology in Europe (Chmielewski *et al.*, 2004; Dose and Menzel, 2004; Estrella *et al.*, 2007). In Germany, cherry bloom of early-maturing cultivars has advanced up to 4.7 days per degree (Chmielewski *et al.*, 2004), and in South-Western France, warmer winters have dramatically affected sweet cherry production (G. Charlot, Balandran, France, 2016, personal communication).

The cherry tree response to warmer winter temperatures differs between cultivars:

- Chilling requirements for overcoming endodormancy may be satisfied in **low- and medium-chill cultivars**, but heat requirements are satisfied too rapidly. Consequently, an advance in bud break and blooming dates is reported that increases the risk of frost damage under early freezing spells (Cannell and Smith, 1986). In addition to higher risks of spring frost damage, earlier flowering dates can lead to a desynchronisation between flowering dates and pollinator activity (Hegland *et al.*, 2008), and also between the flowering dates of varieties that pollinate each other.

- Conversely in **high-chill cultivars**, chilling requirements for overcoming endodormancy may not be entirely satisfied, thus inducing an incomplete dormancy release, which might lead to bud burst delays, low bud burst rate and lack of uniformity of leafing and bloom (Erez, 2000).

In both cases, this would lead to higher flower bud drop, morphological abnormalities and less usable fruits, which could have an important impact on fruit production.

Another aspect of climate change is an increasing frequency of high summer temperatures during flower bud differentiation, which are believed to cause double pistil formation, resulting in double fruit the following year (Micke *et al.*, 1983; **Figure 2.9**). These anomalies are observed around the world including France (G. Charlot, Balandran, France, 2016, personal communication), Spain (G. Lopez-Ortega, Murcia, Spain, 2015, personal communication), Australia (Thomas *et al.*, 2012), Germany (Chmielewski *et al.*, 2017) and Japan (Primack *et al.*, 2009).

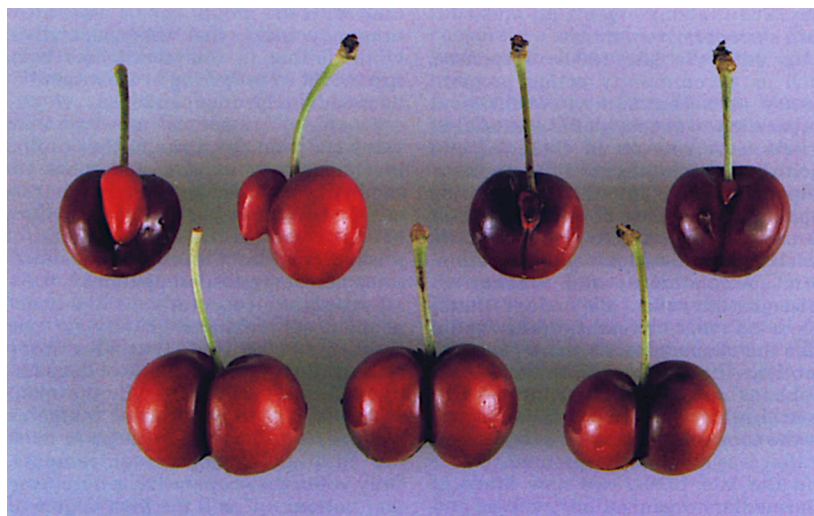


Figure 2.9 | Double fruit due to high temperature in summer
(Micke *et al.*, 1983)

2.2.3 Strategies to tackle fruit losses

In this context of global warming, new strategies are necessary to tackle cherry fruit losses, including the conception of predictive models to predict flowering time to assist growers and breeders, and the creation of new varieties adapted to climate change.

Traits to improve cherry adaptation to climate change are added to the breeding strategies (Quero-Garcia *et al.*, 2017; Wenden *et al.*, 2017):

- Higher heat requirements to produce later bud activation and flowering associated with lower chilling requirements since future warm winters may not allow the full release of dormancy in some regions.

- Breeding cultivars that have lower risks of double fruit where summer temperatures are increasing.

- Self-fertility trait breeding to maximise the chance of cross-pollination with compatible cultivars within an orchard.

A clear biological and mechanistic understanding of physiological processes such as dormancy, flowering and double fruit induction is essential for the development of novel mitigation strategies, including accurate characterisation and utilisation of cherry genetic resources for modifying these traits. Predictive modelling approaches could be powerful tools to assist the breeding strategies in overcoming the complexity of all mechanisms involved in temperature responses of cherries. A number of efforts has been made to model the temperature response during dormancy in perennials. These models are mainly based on the heat and chilling requirements and how temperature is perceived by the trees (see section 2.5.2, page 27). However, no new predictive model for dormancy has been proposed since 1990 (Chuine and Régnière, 2017). Current process-based models of bud dormancy rely on very little information about involved mechanisms mainly because so far, robust molecular or physiological markers of dormancy depth and release are lacking.

2.2.4 THE KEY POINTS TO REMEMBER

- Global warming expectations: increasing temperature and precipitations
- Global warming consequences: frost damages, low bud burst rate, bud drop, morphological abnormalities, productivity drop, double pistil formation, double fruit.
- New strategies to overcome these problems of cherry production: breeding programs to create new varieties adapted to climate change and predictive models. However, **more molecular knowledge is needed to identify mechanisms and marker genes involved in dormancy.**

2.3 Genetic resources

2.3.1 *Prunus* Biological Resources Centre (BRC)

In INRA, the *Prunus* Biological Resources Centre (BRC) maintains *Prunus* collections in two places: Nouvelle-Aquitaine Region and Provence Alpes Côte d'Azur Region. All the collections are managed by the Fruit Experimental Unit (UEA).

In Nouvelle-Aquitaine (Toulence (33) and Bourran (47)), the BRC *Prunus* owns around **1,500 accessions** of *Prunus* species, among them 200 **accessions** resulted from research programs lead by INRA. The *Prunus* collections cover six hectares including 450 m² of

insect-proof greenhouses. Under these facilities the *Prunus* accessions devoted to be distributed (mainly the French reference collections) are secured (virus-free).

The aims of the BRC *Prunus* are:

- To maintain the *Prunus* collections, especially the French reference ones.
- To characterise the *Prunus* collections for phenology, pomology and agronomic traits.
- To promote the exchange of plant material suitable for research, breeding or more generally for old fruit cultivar “amateurs”.
- To collect and disseminate data from the *Prunus* collections.

2.3.2 European Cooperative Programme for Crop Genetic Resources (ECP/GR) *Prunus* working group

The European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR) is a collaborative programme among most European countries and promotes the characterisation and evaluation of plant genetic resources as well as their conservation and utilisation (Iezzoni *et al.*, 2017).

The **ECPGR program** includes six crops and 3 thematic networks. Among them the Fruit Network is composed of three working groups: the *Malus/Pyrus* group, the *Prunus* group and the *Vitis* group. In the ECP/GR *Prunus* working group, experts of 39 different countries cooperate in common objectives. France is an active member of the ECP/GR *Prunus* working group and is in charge of the European *Prunus* **Database (EPDB <http://www.bordeaux.inra.fr/euprunusdb/>)** and contains data for 5,585 cherry accessions, maintained by 42 institutes in different countries (Iezzoni *et al.*, 2017).

2.4 Genomic resources

2.4.1 Genomes

In the last decades, the model species for *Prunus* has been the peach tree and its genome v1.0 and v2.0 have been released in 2013 (Verde *et al.*, 2013) and 2017 (Verde *et al.*, 2017) respectively.

The cherry genome size has been estimated to be between ~**338 Mb** (Arumuganathan and Earle, 1991) and ~**353 Mb** (Shirasawa *et al.*, 2017) about twice the size of the *Arabidopsis thaliana* genome (135 Mb; The Arabidopsis Genome Initiative, 2000). In the last years, several projects have been initiated to sequence sweet cherry genomes. In 2013, the cherry genome sequencing was initiated by the Sweet cherry International Consortium headed by

Amit Dhingra (Washington State University, USA) and Herman Silva (Universidad de Chile, Chile) on 'Stella' cultivar (Carrasco *et al.*, 2013; [Appendix Table 4](#)) but the genome data and its annotation are not available up to now. In 2017, Shirasawa *et al.* published the first cherry genome for the Japanese cultivar 'Satonishiki'.

Our team, through a Sweet Cherry Consortium coordinated by Elisabeth Dirlewanger (INRA, France), has initiated a new sequencing of the sweet cherry cultivar 'Regina' using a combination of sequencing strategies (long reads sequencing with PacBio RSII and optical mapping with BioNano whole-genome maps, the genome annotation is underway (Dirlewanger *et al.*, 2018, [Appendix Table 5](#)).

2.4.2 Cherry transcriptome

The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular events of cells or tissues in development. Transcriptome profiling by RNA-Seq is more efficient and sensitive than microarray analysis or other techniques ([see Appendix Table 6](#) for the list of advantages of RNA-seq compared with other transcriptomic methods, Wang *et al.*, 2009).

To our knowledge, no microarray has been developed for sweet cherry but a few recent transcriptomic analyses were done on cherry:

- Transcriptional dynamics of the developing sweet cherry (*Prunus avium* L.) fruit: sequencing, annotation and expression profiling of exocarp-associated genes (Alkio *et al.*, 2014).
- Transcriptome analysis of dormant flower buds of Chinese cherry (*Prunus pseudocerasus*) using one variety and three time points (Zhu *et al.*, 2015).
- Transcriptional analysis of genes involved in anthocyanin biosynthesis in the red and yellow fruits of sweet cherry (*Prunus avium* L.) (Wei *et al.*, 2015).
- De novo transcriptome assembly of a sour cherry cultivar (Jo *et al.*, 2015).

Generated transcriptomes and gene expression data on cherry provided helpful genetic information for revealing transcriptomic variations during specific events. In 2012, a cDNA bank was built in our team on five different tissues from 'Regina' (buds, leaves, flowers, fruits and branches) and sequenced using the 454-pyrosequencing technology from Roche (454 GS FLX Titanium). Following the sequencing and annotation, a transcriptome database was created and used within the team (Le Dantec *et al.*, 2012). A first RNA-seq analyses was conducted in our team on three time points during dormancy for two

cultivars contrasted for their flowering date 'Regina' and 'Garnet', but data has not been made available yet.

2.4.3 Cherry linkage map and Quantitative Trait Locus (QTL)

Linkage maps are useful tools in the study of plant genetics and breeding. Indeed, they facilitate the identification and characterisation of genomic regions associated with trait(s) of interest and help identifying markers for marker-assisted selection (MAS). MAS is especially advantageous for cherry breeding because of the long juvenile phase.

The first sweet cherry mapping studies begun using random amplified polymorphic DNA (RAPD) markers (Stockinger *et al.*, 1996), and isoenzymes (Bošković and Tobutt, 1998). Later, maps were constructed mainly using single sequence repeat markers (SSRs) (Dirlewanger *et al.*, 2004; Olmstead *et al.*, 2008; Clarke *et al.*, 2009) and more recently, using single nucleotide polymorphisms (SNPs) thanks to a 6K SNP array, developed for sweet and sour cherry in the RosBREED project coordinated by Amy Iezzoni (<https://www.rosbreed.org/>) using the Illumina Infinium® system (Cabrera *et al.*, 2012; Klagges *et al.*, 2013; Peace *et al.*, 2014; Castède *et al.*, 2014; Guajardo *et al.*, 2015; Calle *et al.*, 2018; , Dirlewanger *et al.*, 2018). A new RosBREED cherry 15K SNP array and other genotyping approaches such as Specific-Locus Amplified Fragment (SLAF) (Wang *et al.*, 2015) and Genotyping by sequencing (GBS) approach (Guajardo *et al.*, 2015; Dirlewanger *et al.* 2018) will ease the development of new highly dense linkage maps.

In combination with linkage maps, the detection of chromosome regions containing genes controlling both qualitative and quantitative traits (Quantitative trait loci, QTLs) has been conducted to improve breeding strategies (Salazar *et al.*, 2014) for traits related to phenology (such as bloom time and maturity date), fruit quality (such as fruit weight and firmness) and fruit skin colour (Iezzoni *et al.*, 2017). For bloom time, major QTLs were detected on LG6 for peach and on LG4 for apricot and sweet cherry whereas for maturity date, a major QTL was detected too on LG4 for all three species (Dirlewanger *et al.*, 2012). In sweet cherry one stable QTL with a major effect for chilling requirements and bloom date was detected in the same region but no stable QTL was identified for heat requirements (Castède *et al.*, 2014). Candidate genes underlying the major QTL on **LG4** were investigated and key genes were identified for chilling requirement and bloom date (Castède *et al.*, 2015).

Main candidate genes that colocalise with the major QTL on LG4 are *PavARP4*, *PavEMF2* and *PavPIE1*, which are **involved in chromatin remodelling**, and *PavKS*, *PavKSb*, *PavKSc* and *PavGA2ox* that control **gibberellin homeostasis** (Table 2.6). These results led to question the pathways involved in the regulation of chill requirements and flowering time and

they were the foundation of the guideline of my PhD project.

Gene	Description	Peach gene ID	Transcript start	Sequence length (aa)	Match ID (accession)	Similarity (%)	E-value	Gene ontology – biological process
NUA	Nuclear-pore anchor-like	ppa000061m	6823 622	2039	XP_004293787.1	81	0E0	Negative regulation of flower development; protein import into nucleus
KS	ent-kaurene synthase	ppa001902m	7070 520	745	AEN74904.1	91	0E0	Gibberellin biosynthetic process
KS	ent-kaurene synthase	ppa018714m	7078 032	383	AEN74904.1	77	0E0	Gibberellin biosynthetic process
KS	ent-kaurene synthase	ppa019543m	7087 518	337	AEN74904.1	75	3.19E-111	Gibberellin biosynthetic process
PIE1	Photoperiod-independent early flowering 1	ppa000063m	7488 534	2030	XP_002882790.1	74	0E0	Negative regulation of flower development; chromatin remodelling
GA20ox	Gibberellin 20-oxidase	ppa020361m	7983 415	316	XP_002517548.1	77	1.32E-146	Gibberellin process; oxidation-reduction process
GA2ox	Ggibberellin 2-oxidase	ppa008414m	8581 870	334	ACT99992.1	92	0E0	Gibberellin catabolic process; oxidation-reduction process
EMF2	Embryonic flower 2	ppa002685m	8728 803	646	XP_004300676.1	88	0E0	Vernalization response; negative regulation of flower development; histone methylation
ARP4	Actin-related protein 4-like	ppa006685m	8820 787	401	XP_002275561.2	94	0E0	Long-day photoperiodism, flowering

Table 2.6 | Candidate genes located in the quantitative trait loci (QTLs) on linkage group 4 in sweet cherry (*Prunus avium*, L.) (Castède et al., 2015).

2.4.4 KEY POINTS TO REMEMBER

- The **database for Cherry Genetic Resources** (eucherrydb) contains data for 5,585 accessions, maintained by 42 institutes from 17 countries (<http://www.bordeaux.inra.fr/eucherrydb/>). The *Prunus* BRC provides around 1,300 accessions of stone fruit species including 500 cherry accessions (sweet and sour cherry)
- The cherry genome size has been estimated to be between **~338 Mb** and **~353 Mb**.
- A genic sequence similarity between sweet cherry and peach allows the mapping of our cherry data using the peach genome.
- The first transcriptomic analysis including different varieties displaying different flowering time with a large time-course was carried out within our A3C team.
- Candidate genes underlying **the major QTL on LG4** were investigated and key genes were identified for chilling requirement and bloom dates, including genes involved in **hormone and chromatin remodelling pathways**.

2.5 Dormancy in perennials

2.5.1 Definition

Dormancy is defined as a period of repressed growth essential to protect sensitive tissues under unfavourable climatic conditions.

The growth cessation of active shoot occurs in late summer as the photoperiod and temperature decrease (Lang *et al.*, 1987; Balandier *et al.*, 1993; Campoy *et al.*, 2011). This step marks the first phases of dormancy called (i) paradormancy also named “summer dormancy”, which is a correlative inhibition when the growth inhibition arises from another part of the plant, similarly to apical dominance (Cline and Deppong, 1999). Subsequently, (ii) endodormancy is initiated by lower temperature and shorter photoperiods in autumn (Lang *et al.*, 1987; Heide, 2008) associated with the beginning of the process of cold acclimation (Mølmann *et al.*, 2005; Ruttink *et al.*, 2007; Tanino *et al.*, 2010). At this stage, buds are protected by dark brown external scales (Fadón *et al.*, 2015) rich in phenolic compounds (Codignola *et al.*, 1988; Bílková *et al.*, 1999). Endodormancy is deepened progressively under the control of photoperiod and temperature (Rohde and Bhalerao, 2007; Heide, 2008) until reaching a stage in which bud development becomes unresponsive to growth-promoting temperatures. This inability to resume growth even under favourable conditions strictly defines endodormancy, which is released following exposure to prolonged cold until a threshold is met (fulfilment of chilling requirements). Following the transition from endodormancy to (iii) ecodormancy, bud development is led by warmer temperatures in spring to resume bud growth and achieve flowering (fulfilment of heat requirements; [Figure 2.8](#); from section 2.1.5, page 14).

2.5.2 Chilling and heat requirements

The concept of the chilling requirement (CR) needed to overcome endodormancy was introduced with the chilling hour model (Bennett, 1949; Weinberger, 1950). This information allows to compare cultivars based on their response to cold temperatures and to anticipate budbreak during the winter, which is extremely useful for breeders and growers. The CR parameter is considered to be cultivar specific, highly variable and dependent on the year and the geographical location (Cortés and Gratacós, 2008; Campoy *et al.*, 2011; Tersoglio *et al.*, 2012; Wenden *et al.*, 2017). Moreover, the CRs calculated for a given cultivar may vary in function of different factors: localisation, climatic conditions, yearly variation of temperature, so the CRs assigned in a given location may not be valid elsewhere (Luedeling, 2012). The

chilling hour model assumes that temperatures between 0 and 7.2°C have a chilling effect to overcoming endodormancy, with each hour at temperatures between these thresholds contributing 1 chilling hour. New approaches were developed to solve these problems of variability like the creation of the Utah model by Richardson *et al.* (1974). Presently, the Dynamic model (Erez and Couvillon, 1986), based on the assumption that the accumulation of chill is completed only after a prolonged cold period, is considered one of the best models to calculate chilling requirements for fruit trees.

Contrary to CR, it is still not clear whether cultivars have specific heat requirements (HR) for flowering (Gianfagna and Mehlenbacher, 1985; Alonso *et al.*, 2005) or whether flowering date is determined basically by CRs (Erez and Couvillon, 1987; Ruiz *et al.*, 2007; Campoy *et al.*, 2012). Albuquerque *et al.*, (2008) and Castède *et al.*, (2014) have shown that CRs have much stronger effects on flowering date than HRs in sweet cherry. Same results were found in apricot (Campoy *et al.*, 2012), almond (Sánchez-Pérez *et al.*, 2012) and peach (Okie and Blackburn, 2011).

Impact of photoperiod and temperature on dormancy in woody plants have been largely documented (Allona *et al.*, 2008; Heide, 2008; Tanino *et al.*, 2010) and several studies successfully introduced a day length parameter to their models (Caffarra *et al.*, 2011a, 2011b; Blümel and Chmielewski, 2012; Chmielewski and Götz, 2016; Chuine and Régnière, 2017), thus suggesting that photoperiodic sensitivity is of key importance for dormancy progression, at least for certain perennial species.

These models brought knowledge for explaining dormancy overcoming by temperature but without accounting for biological and physiological processes. There is increasing potential to incorporate recent advances in our understanding of molecular pathways regulating dormancy into phenology models. Research on model organisms such as *Arabidopsis thaliana* or annual crops have shown that phenology models based on molecular mechanisms can now be developed (Nagano *et al.*, 2012; Satake *et al.*, 2013; Kudoh *et al.*, 2016). Therefore the time is now to gain in-depth knowledge on the mechanisms implicated in the regulation of dormancy in perennial species and develop new phenology models.

2.5.3 Mechanisms regulating bud dormancy

Because of the pivotal role of dormancy and temperature perception in temperate trees' development, much work has been done to unravel the mechanisms underlying dormancy progression. Physiological studies including metabolic analyses led to a broad description of the main pathways involved, notably hormone signalling, transporters,

carbohydrates metabolism and oxidative status. More recently, transcriptomic analyses have led to the identification of common molecular pathways regulating bud dormancy in trees. Here, we will present an overview of the pathways identified in these studies, with a focus on molecular findings. The molecular mechanisms controlled by cold temperatures are better documented in the vernalisation and seed dormancy processes in annual plant, which can both be used as a guideline to better understand the general events regulating dormancy in perennials.

2.5.3.1 Main molecular pathways

Similarities have long been recognised between vernalisation, the prolonged exposure to cold temperatures that promotes the floral transition in annual plants like *Arabidopsis thaliana*, and the chilling requirement to release bud dormancy in perennial plants. In both cases, the extended chilling period occurring during winter is used to coordinate developmental events to the appropriate seasonal time. We can therefore argue that common molecular mechanisms might control the response to cold temperatures and the growth resumption under favourable conditions. Crosses between summer and winter annual *Arabidopsis* plants have led to discover two loci responsible for the late flowering and the vernalisation process winter-annual habit in *Arabidopsis*: **FLOWERING LOCUS C (FLC)** and **FRIGIDA (FRI)** (Napp-Zinn, 1987; Lee *et al.*, 1993; Clarke and Dean, 1994; Koornneef Maarten *et al.*, 1994). The presence of a dominant allele of *FRI* raises *FLC* expression to a level that inhibits flowering (Michaels and Amasino, 1999; Amasino, 2005). Vernalisation overcomes the effect of *FRI* by repressing *FLC* expression, and this repression is stably maintained after plants are returned to warm growth conditions allowing plants to flower (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *FLC* is a repressor of flowering that encodes a MADS-box transcriptional regulator and represses the expression of a set of flowering activators, which include **FLOWERING LOCUS T (FT)** and **SUPPRESSOR OF CONSTANS1 (SOC1)** (Michaels and Amasino, 2001). *FT* and *SOC1* are responsible to the activation of a set of genes involved in the formation of floral organ such as *LEAFY* and *APETALA1* (Lee *et al.*, 2008, [Figure 2.10](#)). The vernalisation-mediated repression of *FLC* requires four genes: **VERNALISATION2 (VRN2)**, **VERNALISATION1 (VRN1)**, **VERNALISATION INSENSITIVE3 (VIN3)** and **VIN3-LIKE1 (VIL1)/VERNALISATION5 (VRN5)** (Bastow *et al.*, 2004; Sung and Amasino, 2004). *VRN1* and *VRN2* are responsible for the stable maintenance of the vernalised state by repressing *FLC* following prolonged cold exposure but not for its initial establishment (Gendall *et al.*, 2001; Levy *et al.*, 2002) while *VIN3* is responsible for the initial repression of *FLC* after a long period of cold (Sung and

Amasino, 2004) (Figure 2.10).

Several studies show that *FLC* interacts directly with another MADS-box protein named **SHORT VEGETATIVE PHASE (SVP)** (Pelaz *et al.*, 2000; Hartmann *et al.*, 2000; Honma and Goto, 2001; Li *et al.*, 2008;), which is a key regulator of flowering through the repression of *FT* (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Lee *et al.*, 2007; Figure 2.10). Five other MADS-box are implicated in the control of flowering, acting as floral repressors: **FLOWERING LOCUS M (FLM)/MADS AFFECTING FLOWERING1 (MAF1)**,

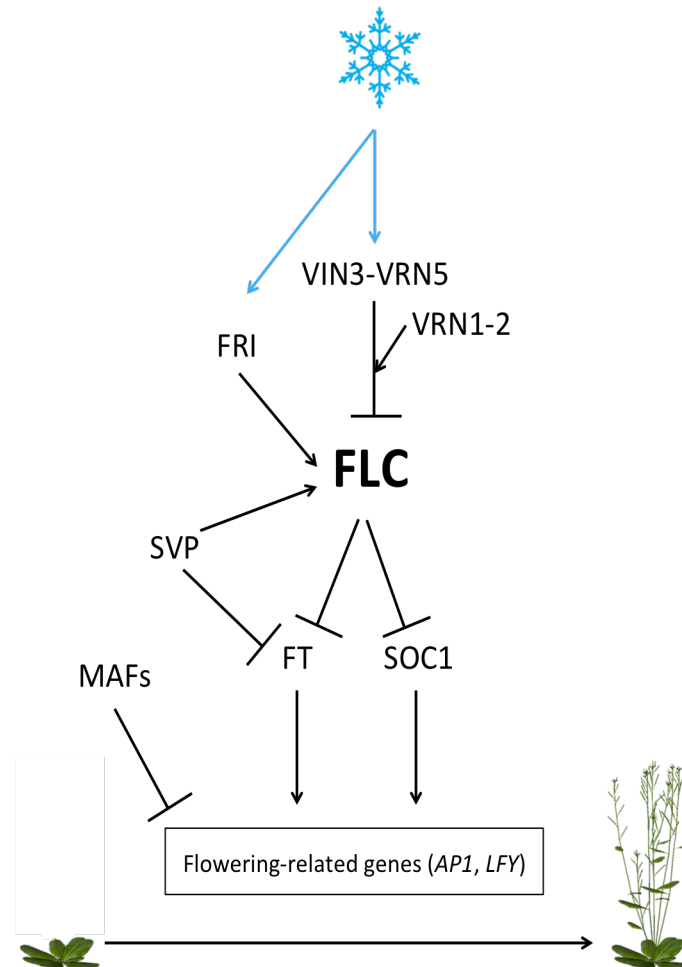


Figure 2.10 | Genes known to be involved in the pathway from vernalisation to flowering

This schema provides an outline of certain flowering pathways. Activation of *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF CONSTANS1 (SOC1)*, in turn, leads to the activation of floral meristem-identity genes such as *LEAFY (LFY)* and *APETALA1 (AP1)*. MADS-box genes, *FLOWERING LOCUS C (FLC)*, *SHORT VEGETATIVE PHASE (SVP)*, *MADS AFFECTING FLOWERING1 (MAF1)/FLM* and *MAF2* repress *FT* and *SOC1*. In winter annual types of *Arabidopsis*, *FRIGIDA (FRI)* is required to up regulate *FLC* to levels that delay flowering. Vernalisation represses the *FRI*-mediated increase in *FLC* expression. The vernalisation-mediated repression of *FLC* is led by *VERNALISATION INSENSITIVE3 (VIN3)* and *VERNALISATION5 (VRN5)*, which are induced by during exposure to cold temperature. The stable maintenance of the vernalised state by repressing *FLC* is mediated by *VERNALISATION1* and *2 (VRN1-2)*.

MAF2, **MAF3**, **MAF4**, and **MAF5** (Ratcliffe *et al.*, 2001; Scortecci *et al.*, 2001; Ratcliffe *et al.*, 2003). *FLM* is similar in amino-acid sequence to *FLC*, thus suggesting a key role of *FLM* as an inhibitor of flowering, although *FRI* and *FLC* account for the majority of the vernalisation response.

In perennials, several studies have led to the discovery that *FT* homologues play an important role in growth cessation, bud set and probably dormancy onset (Hsu *et al.*, 2011; Srinivasan *et al.*, 2012). Only one *FT* homologue was identified in cherry and in peach (Zhang *et al.*, 2015a). Furthermore although *FLC* is the key regulator of vernalisation found in Arabidopsis, no *FLC* orthologous has been found in peach or in sweet cherry (Castède *et al.*, 2014)

Advanced researches in 'Evergreen' (*evg*), a non-dormant peach tree genotype that fails to both cease growth and enter dormancy under dormancy-inducing conditions (Rodriguez *et al.*, 1994), have led to the discovery of **DORMANCY ASSOCIATED MADS-box (DAM)** genes (Bielenberg *et al.*, 2004, 2008). In fact, the deletion of four of the six *DAM* genes are most likely to be responsible for the continuous growth phenotype of the *evg* mutant (Bielenberg *et al.*, 2008; Li *et al.*, 2009). These genes belong to the *SHORT VEGETATIVE PHASE/AGAMOUS-LIKE24 (SVP/AGL24)* subfamily of MADS-box genes in Arabidopsis, which are known to act in the flowering response to environmental signals (Hartmann *et al.*, 2000; Michaels *et al.*, 2003). A positive correlation has been found between the expression of *DAM*-related genes and the bud dormancy status, with up-regulated *DAM*-related genes in dormant buds, while they are down regulated during dormancy release and before bud break (Jiménez *et al.*, 2010b; Yamane *et al.*, 2011; Leida *et al.*, 2012b; Zhu *et al.*, 2015). Following these findings, a key role was proposed for *DAM* genes in establishing and maintaining endodormancy (Li *et al.*, 2009; Horvath *et al.*, 2010; Saito *et al.*, 2015). In sweet cherry, *PavDAM5* and *PavDAM6* co-localize with a major QTL for flowering time in LG1 (Castède *et al.*, 2015). *DAM* genes belong to the same family as *FLC* and *SVP* and it is interesting to note that *FLC* is usually induced by cold temperatures, and then repressed by extended exposure to cold temperatures like some *DAM* genes, suggesting potential similar mechanisms regulating the expression of these key genes for flowering. In addition, a yeast two-hybrid screening demonstrated that Japanese apricot (*Pyrus pyrifolia*) *PmDAM6* can interact in yeast with the homolog of Arabidopsis *SOC1* (*PmSOC1*) (Kitamura *et al.*, 2016). Synchronised expression patterns were detected in *PmDAM6* and *PmSOC1* during dormancy release in flower buds of two contrasted cultivars, suggesting that the dimerisation of *PmDAM6* and *PmSOC1* may play a role in the regulation of dormancy transition and blooming time in Japanese apricot flower buds (Kitamura *et al.*, 2016). Similarly, expression analyses suggest that *DAM* proteins may negatively regulate the expression of *FT* in leafy

spurge and sweet cherry (Hao *et al.*, 2015; Rothkegel *et al.*, 2017). *DAM* genes seem to be good candidates to explain the regulation of dormancy in perennials like *FLC* for vernalisation in *Arabidopsis* but it is probable that other genes play a crucial role in dormancy, since a QTL with a major effect for sweet cherry chilling requirements and bloom date is on LG4 (Castède *et al.*, 2014).

2.5.3.2 Epigenetic regulation of dormancy and flowering

The term ‘epigenetic’ has been traditionally used to refer to heritable changes in gene expression that take place without altering DNA sequence (Wolffe and Matzke, 1999), but it is also used, in a broader sense, to refer to modifications of the chromatin environment (Miozzo *et al.*, 2015). Epigenetic modifications are important for a wide range of processes in plants, including seed germination (Nakabayashi *et al.*, 2005), root growth (Krichevsky *et al.*, 2009), flowering time (He *et al.*, 2003), disease resistance (Stokes *et al.*, 2002) and abiotic stress responses (Zhu *et al.*, 2008). Post-transcriptional modifications of histone proteins and chromatin structure regulates the ability of transcription factors (TFs) to bind DNA and thereby influences gene expression (Lee *et al.*, 1993; Narlikar *et al.*, 2002).

a) Chromatin modifications

Chromatin consist of a compaction of nucleosomes. The nucleosome itself is composed by a core of 8 histones (2x [H2A, H2B, H3 and H4]) linked by the histone H1 and wrapped by DNA (Figure 2.11). Each protein histone has a tail taht can be methylated, acetylated, sumoylated, phosphorylated and ubiquitinated on lysine residues mainly or arginine (Figure 2.11). These processes are reversible.

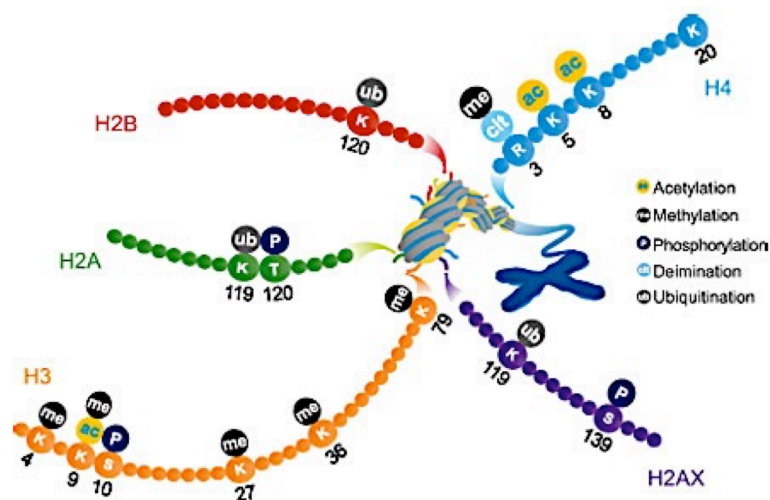


Figure 2.11 | Possible histone modifications

Histone modifications can occur on different amino acids as K=lysine, T=threonine, S=serine, R=arginine.

Some histone modifications, such as tri-methylation of histone H3 at lysine 4 (H3K4me3) are associated with open chromatin and gene activation (Santos-Rosa *et al.*, 2002) while methylation of histone H3 at lysine 27 (H3K27me3) is characterized by a restrictive chromatin environment associated with gene repression (Feng and Jacobsen, 2011). These histone modifications have different spatial arrangements along the gene body (Roudier *et al.*, 2011; **Figure 2.12**) and are reversed by the action of specific enzymes such as histone deacetylases and demethyltransferases. In Arabidopsis, the high levels of *FLC* expression that create the vernalisation requirement are associated with active histone marks, **H3 acetylation (H3ac)**, **H3K4me3** and **H3K36me3** (He *et al.*, 2004; Kim *et al.*, 2009; Tamada *et al.*, 2009; Ko *et al.*, 2010). H3K4 methylation is catalysed by a group of SET-domain containing histone methyltransferases including ***ARABIDOPSIS TRITHORAX1 (ATX1)*** and ***ARABIDOPSIS TRITHORAX-RELATED7 (ATXR7)*** (Alvarez-Venegas *et al.*, 2003; Tamada *et al.*, 2009). The SET domain is important for histone methyltransferase activity, which can catalyse the methylation of several lysine residues in histone H3 and H4 (Lachner and Jenuwein, 2002). **Histone H2B mono-ubiquitination (H2Bub1)** is associated with active gene transcription through the deposition of H3K4me3 and the transcriptional activation of target genes (Wood *et al.*, 2003). H2Bub1 is mediated by a protein complex that contains ***UBIQUITIN-CONJUGATING ENZYME1 (UBC1)***, ***UBC2*** and ***UBC3*** and ***HISTONE MONOUBIQUITINATION1 (HUB1)*** and ***HUB2***. Mutations in either ***HUB1*** or ***HUB2*** result in early flowering and the loss of H3K4me3 enrichment at the promoter region of *FLC* (Cao *et al.*, 2008; Gu *et al.*, 2009).

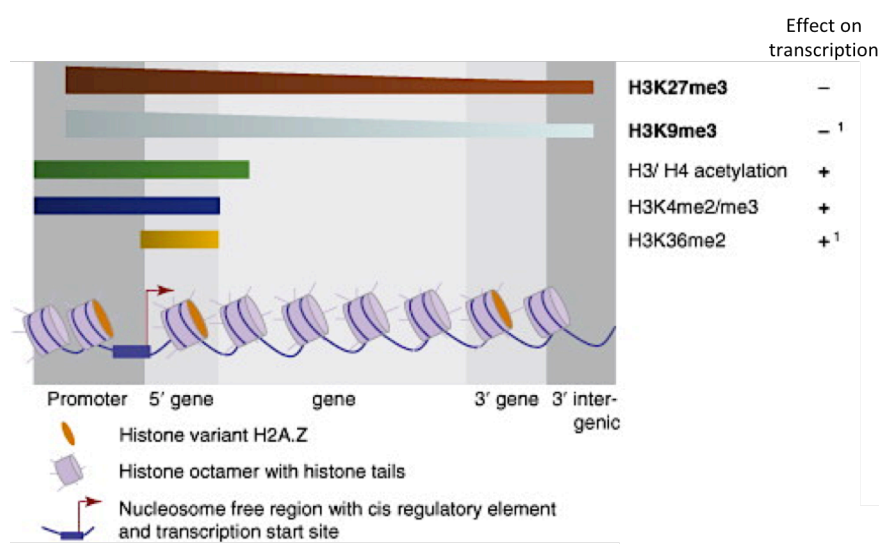


Figure 2.12 | Spatial arrangement of chromatin modifications important for gene expression
 Histone octamers are shown as purple cylinders. The histone variant H2A.Z incorporated by the SWR1 chromatin-remodeling complex is shown as an orange ellipse. The promoter is partially depleted of nucleosomes, which is observed in many constitutively expressed genes. Grey shading highlights the promoter region, the 5' end of the gene, the central gene region, the 3' end of the gene, and the 3' intergenic region. The spatial distribution of covalent histone modifications is indicated relative to these five regions. ¹H3K9me3 and H3K36me2 may have different roles on transcription dependent on their location (Pfluger and Wagner, 2007).

The four key genes mentioned before, *VIN3*, *VRN5*, *VRN1* and *VRN2* (Figure 2.13), mediate the vernalisation-mediated repression of *FLC* through epigenetic mechanisms:

VRN2 encodes a nuclear-localised zinc finger protein and is a homologue of *Suppressor of Zeste 12 (Su(z)12)*, which is a component of Polycomb Repressive Complex 2 (PRC2) (Gendall *et al.*, 2001; Czermin *et al.*, 2002). PRC2 is a methyltransferase complex that is responsible for the repression of gene expression by catalysing the trimethylation of the lysine 27 at the histone 3 (H3K27me₃) through its catalytic component Enhancer of Zeste (E(z)), a SET-domain containing methyltransferase (Cao *et al.*, 2002; Müller *et al.*, 2002; Cao and Zhang, 2004). In *vrn2* mutants, the levels of H3K27 methylation and H3K9 methylation, another repressive histone mark, are not increased at *FLC* locus during the cold exposure (Bastow *et al.*, 2004; Sung and Amasino, 2004; Figure 2.13).

VRN1 encodes a plant-specific DNA-binding protein (Levy *et al.*, 2002). In *vrn1* mutant, the level of H3K27 methylation is increased, but not the level of H3K9 methylation in *FLC* chromatin during and after vernalisation (Bastow *et al.*, 2004; Sung & Amasino, 2004), suggesting that H3K9 and H3K27 methylation can occur independently and *VRN1* is preferentially involved in H3K9 methylation (Figure 2.13).

VIN3 encodes a plant homeodomain (PHD) finger protein known as a component of chromatin remodelling complexes (Bienz, 2006). Mutations in *VIN3* result in a vernalisation-insensitive phenotype and *VIN3* expression is inversely correlated with *FLC* expression during cold exposure. In *vin3* mutant, *FLC* expression is not repressed during vernalisation and vernalisation-mediated histone modifications including H3 acetylation, H3K9 and H3K27 methylation do not occur at *FLC* chromatin (Sung & Amasino, 2004). Finally, *VIN3* and *VIL1/VRN5* form a heterodimer necessary for vernalisation-mediated histone modifications at *FLC* chromatin including the methylation of both H3K9 and H3K27 (Greb *et al.*, 2007).

Histone variants also play roles in the regulation of gene expression. ***PHOTOPERIOD INDEPENDENT EARLY FLOWERING1 (PIE1)*** encodes the catalytic subunit of the chromatin-remodelling complex SWR1, which incorporates the histone variant H2A.Z into nucleosomes, and has been identified as a positive regulator of *FLC* expression (Noh and Amasino, 2003; Choi *et al.*, 2007). Mutations in *PIE1* result in the loss of H2A.Z from *FLC* chromatin leading to reduced levels of *FLC* expression and early flowering (Deal *et al.*, 2007; Figure 2.13). Other subunits of the SWR complex have been identified in Arabidopsis including *ACTIN-RELATED PROTEIN6 (ARP6)*, *ARP4*, *SUPPRESSOR OF FRIGIDA3 (SUF3)* and *EARLY IN SHORT DAYS1 (ESD1)* (Noh and Amasino, 2003; Choi *et al.*, 2005; Deal *et al.*, 2007). Mutations in these genes also suppress *FLC* expression and thus lead to early flowering. All these results suggest a fine epigenetic (histone marks) regulation of flowering through the repression or activation of *FLC* during vernalisation.

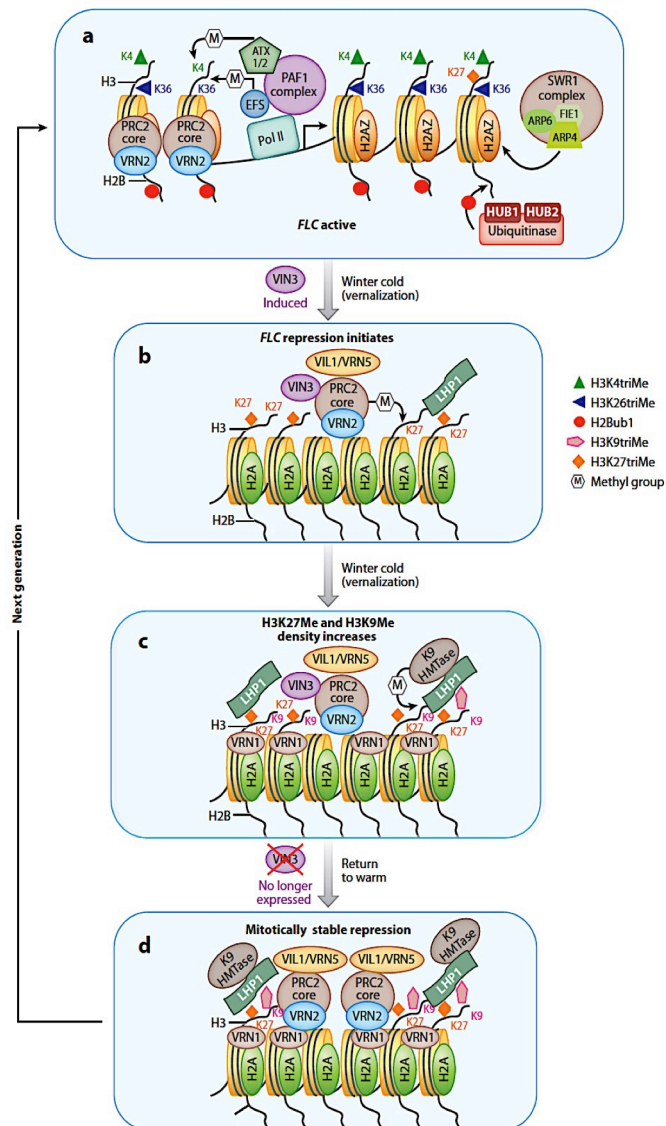


Figure 2.13 | Vernalisation-mediated changes in FLC chromatin

(a) Prior to cold exposure, *FLC* is actively expressed. The complexes that maintain this active chromatin conformation include the PAF complex, which methylates histone 3 tails at lysine 4 and 36 (H3K4me3 and H3K36me3), a SWR1-like complex, which deposits a histone 2A variant in the nucleosomes of *FLC* chromatin, and H2B ubiquitinases like HUB1 and HUB2 that ubiquitinate histone 2B tails (H2Bub1). Although *FLC* is in an active state, there are repressive complexes present such as Polycomb Repression Complex 2 and some degree of lysine 27 methylation of histone 3 (H3K27me3—a repressive modification) (b) During cold exposure, *FLC* repression is initiated. *VIN3* is induced, *VIN3* and *VIL1/VRN5* associate with the Polycomb complex, the density of repressive chromatin modifications such as lysine 27 methylation of histone 3 increases, and repressors such as LIKE HETEROCHROMATIN PROTEIN 1 (*LHP1*) assemble on *FLC* chromatin. (c) As vernalisation proceeds, the density of repressive modifications, particularly H3K27triMe and lysine 9 methylation of histone 3 (H3K9me3; mediated by an unknown H3K9 methyltransferase (*HMTase*)) increases. (d) Eventually, a mitotically stable state of repression that no longer requires *VIN3* is achieved. This mitotically stable state is likely to involve positive feedback loops in which the repressive chromatin modifications serve to recruit the chromatin-modifying complexes including *VRN1* to maintain a repressive state. As the *FLC* locus passes to the next generation, the active chromatin state represented in (a) is re-established (Kim *et al.*, 2009).

In perennials, the global epigenome studies are quite recent and only a few results emerge from the literature mainly based on the regulation of *DAM* genes.

The regulation of *PpeDAM* gene silencing during the transition from dormancy to dormancy release in peach is under the control of the repressive histone mark H3K27me3 (De la Fuente *et al.*, 2015). Conversely, the positive regulation of *PpeDAM6* in peach dormant buds is associated by the presence of active histone mark H3K4me3 and H3ac modifications around the translation start site (Leida *et al.*, 2012a), similarly to results for *DAM1* in leafy spurge (Horvath *et al.*, 2010). Putative histone deacetylases (*HDA14* and *HDA08*), histone lysine methyltransferase (*SUVR3*), and *HUB2* involved in histone ubiquitination were up regulated during the transition active growth to dormancy in hybrid aspen (Karlberg *et al.*, 2010). Several genes belonging to the Trithorax family of factors counteracting the repressive effect of Polycomb complex were down regulated at this same period (Karlberg *et al.*, 2010). In chestnut (*Castanea sativa*), the expression of *CsHUB2* and *CsGCN5L* coding for putative histone mono-ubiquitinase and histone acetyl transferase, respectively, were higher in dormant buds (Santamaría *et al.*, 2009 and 2011). Saito *et al.*, (2015) have shown that a reduction of the activation histone mark (H3K4me3) contributed to the reduction of *PpMADS13-1* expression towards endodormancy release in Japanese pear. In contrast, the repressing histone mark (H3K27me3) in *PpMADS13-1* locus was quite low in endodormancy phase transition. Additionally, the loss of histone variant H2A.Z also coincided with the down-regulation of *PpMADS13-1* (Saito *et al.*, 2015). In sweet cherry, several candidate genes identified as homologues of Arabidopsis genes involved in chromatin remodelling or modification complexes were located in major QTLs for flowering and dormancy, such as *EMF2*, *PIE1* and *ARP4* (Castède *et al.*, 2014, 2015).

b) DNA methylation

DNA methylation is important for the control of various biological processes including the timing of flowering regulation in Arabidopsis (Burn *et al.*, 1993; Finnegan *et al.*, 2000). In plants, methylation of cytosines at the carbon 5 position from S-adenosyl-L-methionine (SAM), is a characteristic feature of many eukaryotic genomes. One way to actively reprogram the epigenome is to remove methylated cytosines. The Arabidopsis genome encodes four bifunctional helix-hairpin-helix **DNA glycosylases** and **AP lyases** named **REPRESSOR OF SILENCING1 (ROS1)**, **DEMETER (DME)**, **DEMETER-LIKE2 (DML2)** and **DEMETER-LIKE3 (DML3)** which recognise and remove methylated cytosines (Feng *et al.*, 2010). A link has been found between DNA methylation and histone mark in mammalian genome; in fact, unmethylated CpG (i.e. loci with high content of CG dinucleotides) are specifically bound by CFP1 (CXXC finger protein-1), which in turn recruits the histone H3K4

methyltransferase SETD1 (SET domain containing-1) (Thomson *et al.*, 2010). Thus, suggesting that H3K4 methylation with the exclusion of DNMT3 from CpG islands may explain how promoters remain unmethylated. In addition, Roudier *et al.*, (2011) shown that the change of chromatin state is due to the combination of several epigenetic marks and not only the action of one. They demonstrated that the Arabidopsis epigenome contains four predominant chromatin states (Figure 2.14):

- 1) CS1: H3K4me2-3, H3K9me3, H3K36me3 and with a medium level of DNA methylation is associated with active gene expressions.
- 2) CS2: H3K4me2, H3K27me2-3 and without DNA methylation is associated with repressed gene expressions.
- 3) CS3: H3K9me2, H4K20me1 and with a high level of DNA methylation is associated with the presence of transposable elements.
- 4) CS4: No modification is associated with introns.

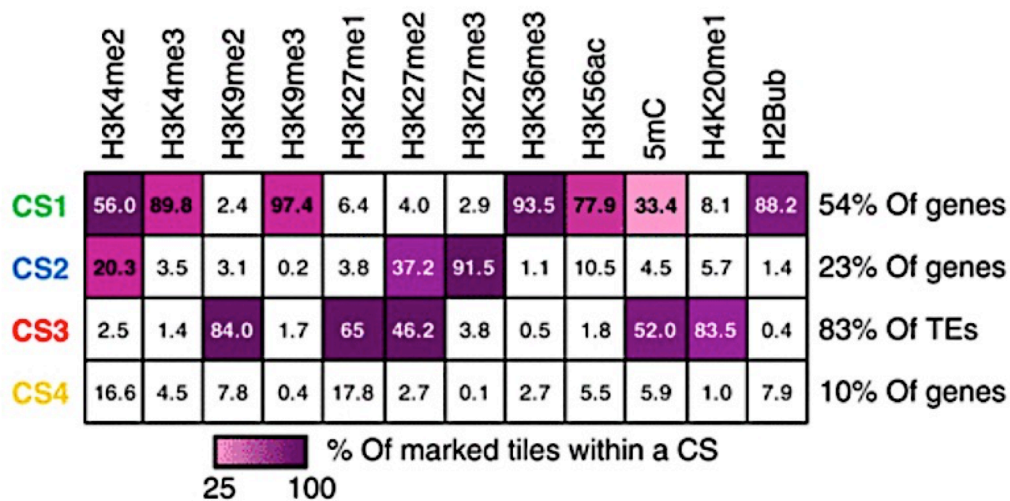


Figure 2.14 | Four predominant chromatin states (CS)

The distribution of the 12 chromatin marks over the four CS is indicated as a heat map for values ranging from 25% (light purple) to 100% (dark purple). The degree of homogeneity of each CS is indicated by the percentage of tiles assigned to it that are associated with each of the 12 chromatin marks (numbers inside cells) (Roudier *et al.*, 2011).

In perennials, the presence of DNA methylation has been established to be involved in the dormancy process. In chestnut, increased methylation status of cytosine coincided with bud set and decreased methylation with bud burst (Santamaría *et al.*, 2009). In addition, a putative DEMETER-like DNA glycosylase, associated with removal of DNA methylation, is down regulated during the transition from active growth to dormancy in hybrid aspen

(Karlberg *et al.*, 2010) highlighting the importance of the presence of DNA methylation on dormancy-related genes during dormancy onset. In addition, it has been shown in poplar that the overexpression of a DEMETER promotes early apical bud maturation (Conde *et al.*, 2017a) and the reactivation of growth in the apical shoot during bud break process in spring is preceded by a progressive reduction of genomic DNA methylation in apex tissue (Conde *et al.*, 2017b). Therefore confirming the important role of DNA methylation in the regulation of dormancy.

Recently, Rothkegel *et al.*, (2017) have demonstrated that the decrease of the expression of *PavMADS1* MAD-box gene in sweet cherry (*PpaDAM3* in peach) was correlated with an increase of DNA methylation and abundance of matching small interference RNAs (siRNAs) in the promoter of *PavMADS1* after dormancy release. However, the levels of methylation in *PavMADS2* (*PpaDAM5* in peach) were not related to the transcript levels and no siRNAs were associated. Thus, suggesting that DNA methylations and siRNAs appear to be involved in the silencing of *PavMADS1* during cold accumulation and dormancy release in sweet cherry and probably other pathways are implicated in the silencing of *PavMADS2* expression.

2.5.3.3 Hormonal regulation of dormancy

The hormonal regulation of seed dormancy is relatively well understood and often used as a model for bud dormancy. Plant hormones that act in correlative inhibition and plant growth in general include abscisic acid (ABA) and gibberellic acid (GA), respectively. But auxin, cytokinins, ethylene, jasmonic acid (JA) and salicylic acid (SA) are also known to regulate plant development processes, including dormancy.

a) ABA is positive regulator of dormancy induction and a negative regulator of dormancy release

The role of ABA controlling seed dormancy has been well established, however, its role in regulating bud dormancy is not well known. In Arabidopsis seeds, overexpression of certain genes related to ABA synthesis present an increase of ABA content resulting in enhanced seed dormancy or delayed germination (Frey *et al.*, 1999; Thompson *et al.*, 2000; Lindgren *et al.*, 2003; Nambara and Marion-Poll, 2003; Holdsworth *et al.*, 2008) while dormancy is reduced in mutants presenting an ABA deficiency (Nambara *et al.*, 1998; González-Guzmán *et al.*, 2004; Lefebvre *et al.*, 2006). The role of ABA in bud dormancy was firstly established by exogenous ABA application. Delayed bud break was reported following

ABA application in birch (Rinne *et al.*, 1994), sour cherry (Mielke and Dennis, 1978), grapevine (Zheng *et al.*, 2014) and pear (Li *et al.*, 2018). In autumn when temperature and photoperiod decrease (paradormancy), several studies have shown a marked increase in ABA content resulting in cessation of shoot growth, promotion of apical buds set and induction of bud dormancy (Rohde *et al.*, 2002). During endodormancy, ABA levels reach their maximum in apical buds while decreasing ABA content is associated with growth resumption (Rohde *et al.*, 2002; Rohde and Bhalerao, 2007; Ruttink *et al.*, 2007; Wang *et al.*, 2016; Tuan *et al.*, 2017; Li *et al.*, 2018; Tylewicz *et al.*, 2018). ABA levels in early and late blooming apple buds differed in pattern of accumulation, but not in concentration (Swartz and Powell, 1981), whereas early Japanese apricot cultivars contained less ABA than late cultivars during dormancy (Wen *et al.*, 2016). Thus, suggesting a dose- and specie-dependent control of dormancy.

Multiple studies conducted in model species have led to the description of key actors involved in ABA pathway (Figure 2.15). The ABA biosynthesis starts with the epoxidation of zeaxanthin into antheraxanthin and violaxanthin by zeaxanthin epoxidase (ZEP) in plastids (Koornneef *et al.*, 1982; Duckham *et al.*, 1991; Marin *et al.*, 1996). Violaxanthin is then converted to 9-cis-violaxanthin and 9-cis-neoxanthin, which are cleaved by **9-cis-epoxycarotenoid dioxygenase** (encoded by ***NCED***) producing xanthoxin (Schwartz *et al.*, 2003; Leng *et al.*, 2014). Xanthoxin is transported to the cytosol, where it is converted to ABA by an alcohol dehydrogenase (encoded by *SDR1*; Cheng *et al.*, 2002; González-Guzmán *et al.*, 2002) and by an aldehyde oxidase 3 (encoded by *AAO3*; Seo *et al.*, 2000; Bittner *et al.*, 2001).

The recognition of ABA is led by **PYR/PYL/RCAR** receptor proteins (Ma *et al.*, 2009; Park *et al.*, 2009; Figure 2.15):

- In presence of ABA, PYR/PYL/RCARs bind to **PP2Cs** (protein phosphatases such as *ABI1* and *ABI2*) and inhibit phosphatase activity, leading to the activation of **SnRK2s** (protein kinases).

- In absence of ABA, PP2Cs bind to SnRKs and inhibit kinase activity.

PP2Cs and the SnRK2s act as negative and positive regulators of ABA signalling, respectively (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Schweighofer *et al.*, 2004; Wasilewska *et al.*, 2008; Rubio *et al.*, 2009). The overexpression of *ABSCISIC ACID INSENSITIVE 5 (ABI5)* in Arabidopsis delayed flowering through the up-regulation of the floral repressor *FLOWERING LOCUS C (FLC)* (Wang *et al.*, 2013). A similar regulatory mechanism has been reported for the overexpression of *ABSCISIC ACID INSENSITIVE 4 (ABI4)* (Shu *et al.*, 2015). In simple terms, ABA seems to regulate the floral transition by acting on the expression of *FLC* in Arabidopsis.

The ABA inactivation or catabolism is a decisive step to control its balance within the plant. ABA can be hydroxylated by ABA 8'-hydroxylase (encoded by **CYP707A**), which is considered as the key catabolic enzyme in ABA inactivation for controlling ABA level, to **ABA phaseic acid (PA)** (Kushiro *et al.*, 2004; Saito *et al.*, 2004; **Figure 2.15**). This reaction has been reported to be reversible (Jadhav *et al.*, 2008). In transgenic ABA 8'-hydroxylase RNAi barley, lower expression of ABA 8'-hydroxylase occasioned a higher ABA content in grains and increased dormancy (Gubler *et al.*, 2008). Finally, PA can be catalysed by a soluble PA reductase (PAR) to **ABA dihydrophaseic acid (DPA)**; Weng *et al.*, 2016; **Figure 2.15**). Further oxidation products of 8'-hydroxy ABA have been identified (Zaharia *et al.*, 2004) and ABA can be conjugated with glucose to **ABA-glucose ester (ABA-GE)** by UDP-glycosyltransferases (UGT). ABA-GE has been suggested to be a form of ABA storage or a potential transport form of ABA through the plant (Sauter *et al.*, 2002) but there is no confirmation that ABA-GE may be cleaved to release ABA within the plant. An *Arabidopsis* UDP-glycosyltransferase encoded by *UDP-glycosyltransferase 71B6* has been found to preferentially glycosylates ABA into ABA-GE (Priest *et al.*, 2006).

Variations in ABA content have been reported to be accompanied by contrasted expression patterns for genes involved in ABA signalling pathways, including the induction of genes related to ABA biosynthesis during dormancy onset (*ABA1*, *NCED3*, and *ABA2*; Arora *et al.*, 2003; Ruttink *et al.*, 2007). Several studies reported that the up-regulation of *NCEDs* also allows to maintain dormancy (Fennell *et al.*, 2015; Wang *et al.*, 2016). After chilling requirements are satisfied (ecodormancy onset), *CYP707A* genes are up-regulated (Zhang *et al.*, 2015b; Wang *et al.*, 2016; Tuan *et al.*, 2017) and biosynthesis genes are down-regulated resulting in lower ABA content (Zheng *et al.*, 2014; Chao *et al.*, 2017). Interestingly, mutants impaired in their ABA response exhibit bud abnormalities and compromised photoperiodic control of bud dormancy but they still cease growth and set buds (Ruttink *et al.*, 2007; Tylewicz *et al.*, 2018). Recent studies suggest that ABA may mediate bud dormancy through the control of cell-to-cell transport (Tylewicz *et al.*, 2018).

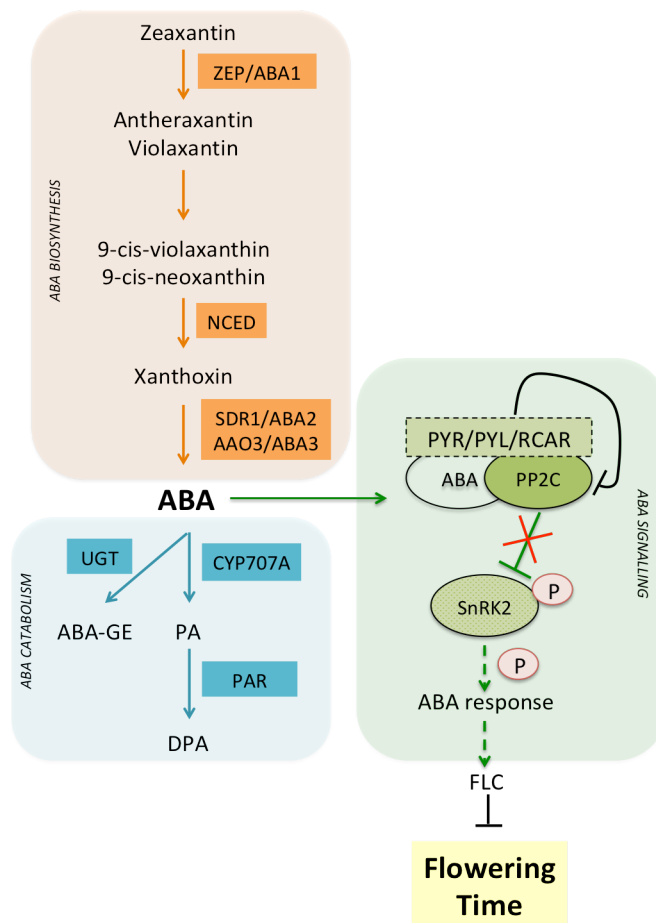


Figure 2.15 | ABA pathway involved in flowering repression through the activation of *FLC*

b) GAs are involved in dormancy release

Bioactive gibberellins modulate growth and development throughout the whole life cycle of plants. GA biosynthesis and catabolism pathways have been studied extensively. 136 GAs have been identified in higher plants and fungi (MacMillan, 2002) but many of these GAs are intermediates or catabolites of bioactive GAs and only a few are biologically active such as GA₁, GA₃, GA₄ and GA₇ (Hedden and Phillips, 2000).

The biosynthesis of GA can be divided into three stages: 1) Biosynthesis of ent-kaurene from geranyl geranyl diphosphate (GGDP) in proplastids by **ent-copalyl diphosphate synthase (CPS)**, encoded by *GA1*; Sun and Kamiya, 1994) and **ent-kaurene synthase (KS)**, encoded by *GA2*, Yamaguchi *et al.*, 1998). 2) Conversion of ent-kaurene to produce GA₁₂ by **ent-kaurene oxidase (KO)**, encoded by *GA3*; Helliwell *et al.*, 1998) and ent-kaurenoic acid oxidase (KAO). 3) bioactive GAs, including GA₁ and GA₄, are catalysed by 2-

oxoglutarate-dependent dioxygenases, **GA 20-oxidases (GA20ox)**, encoded by *GA5*; Phillips *et al.*, 1995) and **GA 3-oxidases (GA3ox)**, encoded by *GA4*; Chiang *et al.*, 1995; **Figure 2.16**).

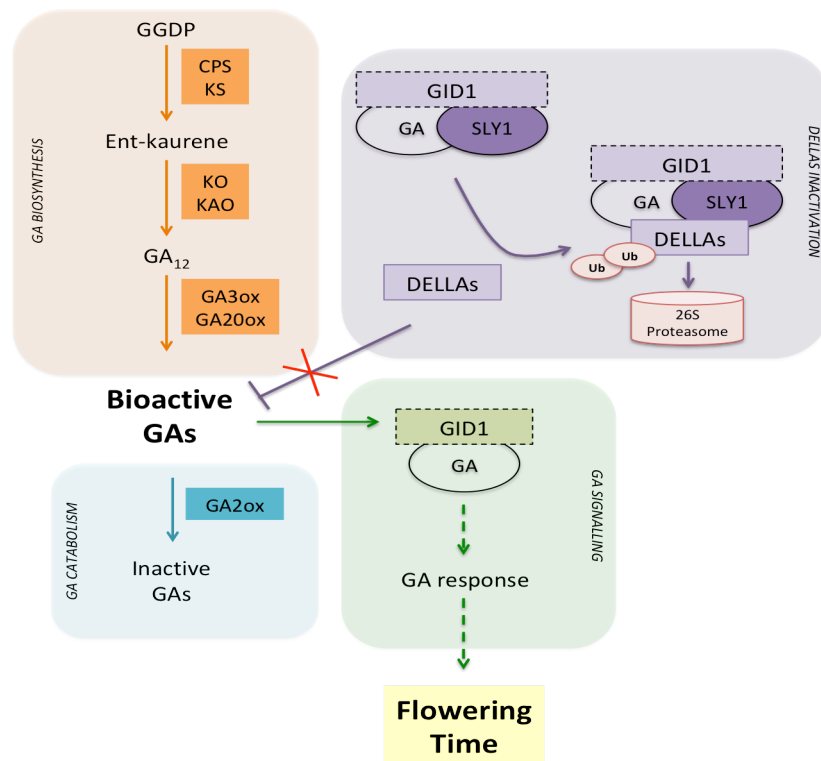


Figure 2.16 | GA pathway involved in flowering time

A balance between biosynthesis and deactivation/catabolism of GA can affect the level of bioactive GAs. **GA2-oxidases (GA2ox)** was found to convert active GAs to inactive forms (Sun, 2008; **Figure 2.16**). A feedback regulation of GA by GA has been revealed by exogenous application of GA and mutants where the expression of biosynthesis genes (*GA3ox* and *GA20ox*) is down-regulated whereas catabolism genes were up-regulated (*GA2ox*; Thomas *et al.*, 1999). Additionally to the effects of GA on seed dormancy and germination, GA is known to promote flowering in several species mediated by two flowering time genes, **LEAFY (LFY)** and **SUPPRESSOR OF OVEREX-PRESSION OF CO1 (SOC1)** (Blazquez *et al.*, 1998; Moon *et al.*, 2003). Five **DELLA** protein genes (*RGA*, *GAI*, *RGA-Like1 (RGL1)*, *RGL2* and *RGL3*) were found in Arabidopsis to repress GA response (Tyler *et al.*, 2004). Removing *RGA* and *GAI* functions in the *ga1-3* background have rescued vegetative growth and floral initiation, indicating that DELLAs are the main GA repressors (Dill and Sun, 2001; King *et al.*, 2001). Several studies demonstrated that DELLAs were directly bound to the receptor **GID1 (GIBBERELLIN INSENSITIVE1)** identified to be the major GA receptor (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2007; Feng *et al.*, 2008) and to the F-box protein **SLY1 (SLEEPY1)** which recruit and target specific proteins

for ubiquitination and degradation through the 26S proteasome (Gagne *et al.*, 2002; Smalle and Vierstra, 2004). So, SLY1 and GID1 proteins may activate the GA pathway by targeting DELLA proteins (GA signalling repressors) for ubiquitination and degradation (Figure 2.16)

GA treatments have a controversial effect on dormancy and bud break. In fact, exogenous application of GA in grapevine inhibits shoot growth and delay bud burst (Weaver, 1959) as well as in peach, apricot, almond, sweet cherry, and apple (Hoad, 1983). While, a dormancy-releasing effect was observed on crown of leafy spurge (Shafer and Monson, 1958) as well as in birch (Eagles and Wareing, 1964), Japanese apricot (Zhuang *et al.*, 2013) and in poplar (Rinne *et al.*, 2011). Interestingly, the application of GA₄ in poplar induced bud break but not GA₃ (Rinne *et al.*, 2011). Nonetheless, Yamaguchi *et al.*, (2014) exposed the complex effect of GA on Arabidopsis flowering and this could explain the controversial results found in perennials after GA treatments.

The highest levels of GA₁ and GA₃ were found in dormant buds during endodormancy release and decreased afterwards (Luna *et al.*, 1990; Wen *et al.*, 2016). These results were supported by transcriptomic analysis where the expression of GA 3-oxidases (GA3ox) and GA 20-oxidases (GA20ox), involved in the biosynthesis of active GAs, were up regulated under chilling treatment (Rinne *et al.*, 2011) and during dormancy release (Bai *et al.*, 2013; Wen *et al.*, 2016). On the opposite, expression of GA 2-oxidases (GA2ox), involved in the catabolism of GA, was up regulated during dormancy and dormancy release in Japanese apricot buds (Yamane *et al.*, 2011) but during ecodormancy as well in Japanese pear (Bai *et al.*, 2013) and Chinese cherry (Zhu *et al.*, 2015).

c) GA counteracts ABA effects

In general, GA and ABA play an antagonistic role in promoting and inhibiting plant growth, respectively. Multiple studies on ABA and GA pathway demonstrated that ABA and GA affected their metabolism each other. In fact, higher ABA level was found in seeds of mutant presenting a GA deficiency (*ga1-3*) (Oh *et al.*, 2007), whereas seeds of mutant presenting an ABA deficiency (*aba2*) exhibited an increase of GA biosynthesis by activating GA3ox1 and GA3ox2 expression (Seo *et al.*, 2006).

In perennials, the ratio ABA/GA increased during endodormancy and decreased during dormancy release in sweet cherry flower buds (Duan *et al.*, 2004). In tea plants (*Camellia sinensis* (L.) O. Kuntze), CsGID1 was down regulated under different ABA treatments, suggesting that ABA inhibits the GA response by suppressing the transcript abundance of GA receptors (Yue *et al.*, 2017). In contrast to the effects of ABA on the expression of GA-related genes, most of the genes involved in the ABA synthesis pathway were repressed by GA₃ treatment (Yue *et al.*, 2017). These results suggest that the ABA and

GA feedback found in tea plants is similar to the Arabidopsis mechanism; however, it is also important to confirm similar mechanism in another perennial plant.

d) GA and ABA crosstalk with other hormones and their implication in perennial dormancy

Ethylene is involved in many aspects of the plant life cycle, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (reviewed in Wang *et al.*, 2002). Ethylene treatment of grapevine increased bud break (Ophir *et al.*, 2009). Ethylene has also been reported to be involved in bud dormancy induction in poplar (Ruttink *et al.*, 2007) and release (Ophir *et al.*, 2009), possibly through a crosstalk with ABA. Indeed, Ruttink *et al.*, (2007) showed that ethylene biosynthesis and signalling appear to precede ABA during the onset of dormancy and the presence of ethylene was crucial for ABA accumulation. In addition, ethylene-insensitive trees presented an appearance of shrub due to reduced apical dominance, thus suggesting that the ethylene pathway interacts with the pathways of ABA and auxin (Ruonala *et al.*, 2006).

Auxin plays an important role in almost every aspect of plant development (reviewed in Leyser, 2002). Auxins were reported to be present in different concentration in buds throughout dormancy progression. Dormancy onset and cold treatment induces a reduction in auxin while quantity of auxins rises in ecodormancy until budburst as shown in hazel buds (*Corylus avellana* L.; Rodríguez and Sánchez, 1986) and grapevine (*Vitis vinifera* L.; Aloni *et al.*, 1990). Similarly, levels of the auxin indole-3-acetic acid (IAA) decline gradually under SD in parallel with increasing endodormancy in bay willow (*Salix pentandra*; Olsen *et al.*, 1997) and silver birch (*Betula pendula*; Li *et al.*, 2003). In addition, it has been shown in apple that genes related to auxin transport are major regulators of dormancy (Porto *et al.*, 2015).

Transcriptomic analyses of dormant buds suggest that brassinosteroid-, salicylic acid- and jasmonic-acid-associated genes are differentially regulated during dormancy (Howe *et al.*, 2015). EARLY BUD BREAK1 (EBB1), a bud break inducer transcription factor, is induced following a cytokinin treatment (Yordanov *et al.*, 2014).

2.5.3.4 Other important pathways involved in dormancy

a) Reactive oxygen species

Plants are subjected to multiple stresses such as abiotic and biotic stresses. In response to these stresses, plants are able to produce reactive oxygen species (ROS) and to control their amount through detoxification systems (Mittler *et al.*, 2011). Under natural conditions, ROS accumulation has been associated with bud break in perennials. Levels of

Hydrogen peroxide (H₂O₂), one of the main ROS, increase gradually in flower buds of Japanese pear and peak when bud dormancy is broken (Kuroda *et al.*, 2002). Hydrogen peroxide accumulation is also correlated to chill accumulation in Japanese pear (Kuroda *et al.*, 2002) and Japanese litchi (*Litchi chinensis* Sonn; Zhou *et al.*, 2012). Additionally, several studies suggest that exogenous application of H₂O₂ substitute chilling suggesting that ROS are involved in dormancy release, notably through the activation of bud breaking genes (Kuroda *et al.*, 2002; Pérez and Burgos, 2004; Pérez *et al.*, 2008). Antioxidant defence and detoxification mechanisms involving catalase (CAT), glutathione peroxidase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidase superfamily proteins, were up-regulated during dormancy release (Scalabrelli *et al.*, 1991; Or *et al.*, 2000; Halaly *et al.*, 2008; Leida *et al.*, 2010; Prassinis *et al.*, 2011; Vergara *et al.*, 2012; Viti *et al.*, 2012; Bai *et al.*, 2013; Zhuang *et al.*, 2013). The ratio between reduced and oxidised glutathione (GSH, GSSG) involved in ROS scavenging appears to be determinant during the process of dormancy release (Siller-Cepeda *et al.*, 1992; Wang and Faust, 1994; Kocsy *et al.*, 2001; Bartolini *et al.*, 2006; Zanol *et al.*, 2010), thus further indicating that ROS play an important role in dormancy.

b) Cell-to-cell transport

Callose deposition is governed by the joint action of 1,3-b-glucansynthase (callose synthase) and 1,3-b-glucanase (glucan hydrolase family 17; GH17) (Levy *et al.*, 2007; Simpson *et al.*, 2009). During dormancy, plasmodesmata in buds are blocked by callose deposition leading to an arrest in cell-to-cell transport (Rinne and van der Schoot, 1998). Plasmodesmatal connections can be re-established by degradation of callose by 1,3-b-glucanases (Rinne *et al.*, 2011) and this process of degradation has been shown during dormancy release (Leubner-Metzger, 2003). In poplar, 1,3-β-d-glucanases are induced by chilling and also by GA_s treatment (Rinne *et al.*, 2011). Recently, Tylewicz *et al.*, (2018) reported that the dormant period is due to loss of access to growth promoting signals by communication blockage of plasmodesmata and this process is ABA dependent.

c) Carbohydrates

Changes in carbohydrate dynamics were linked to changes in dormancy status in sweet cherry with a degradation of starch into soluble sugars during dormancy onset and an increase in starch just before budburst (Kaufmann and Blanke, 2017). Compatible trends were found for soluble sugars in walnut tree (*Juglans* spp) with an increase between autumn and winter followed by a significant decrease between winter and spring (Charrier *et al.*, 2017). Furthermore, genes involved in starch degradation were up regulated in the cambial

meristems during endodormancy in poplar (Schrader *et al.*, 2004). In addition in Japanese apricot, Zhuang *et al.*, (2015) showed that sucrose was synthesised during GA₄-induced dormancy release, thus confirming the link between soluble sugars content and end of endodormancy but also the link between sugar and phytohormone pathway.

d) Photoperiod

For several decades, photoperiod has been known to govern growth cessation of many trees in temperate climates (Nitsch, 1957) where leaves perceive photoperiod and emit a signal to the apex, where inactivity is installed (Hemberg, 1949; Wareing, 1956). *FT* and *CO* homologues (mediators of short-day signals for growth cessation) were overexpressed in transgenic aspen (*Populus tremula P. tremuloides*), leading to continuous growth upon exposure to short days (Böhlenius *et al.*, 2006) but it was showed that the growth cessation and dormancy induction in apple and pear were only under the control of temperature independently of the photoperiod (Cook *et al.*, 2005; Heide and Prestrud, 2005). Heide (2008) confirmed that dormancy in *Prunus* species, including cherry, is mainly controlled by temperature and the photoperiodic response of the *Prunus* species is highly temperature dependent.

2.5.4 Cold acclimation

When studying dormancy, it is important to consider interactions with cold acclimation and hardiness that occur in response to cold temperatures as well. Cold acclimation is promoted within a short period of time, one or two days, by short photoperiod and low but non-freezing temperature (Guy *et al.*, 1985; Hong *et al.*, 1992; Lin and Thomashow, 1992; Monroy *et al.*, 1993), with the stimulation of cold-protective proteins for surviving the colder temperatures. Cold acclimation can also be induced by endogenous cues such as ABA (Rinne *et al.*, 1998; Junttila *et al.*, 2002).

Cold acclimation is often associated with **changes in plasma membrane composition**, including enhanced fatty acid desaturation in membrane lipids (Gusta and Wisniewski, 2013), and a **decrease in water content and osmotic potential** within buds and stem in autumn in order to prevent tissue damages by ice formation (Wisniewski *et al.*, 2003). Moreover, higher sugar contents (total soluble sugar, reducing sugars and sucrose) were quantified in bark of acclimated sweet cherry trees compared to non-acclimated trees (Turhan and Ergin, 2012), this allowing to prevent ice formation by depressing the freeze point (Wisniewski *et al.*, 2003) (Figure 2.17). In addition to metabolism and growth modifications during the process of cold acclimation, it was shown that multiple cold

signalling and regulatory pathways are rapidly induced by cold. Dehydrins are the most abundant protein involved in cellular protection during dehydration in woody plants (Welling and Palva, 2006) in response to low and freezing temperatures (Welling *et al.*, 2004). Several dehydrin genes contain the **LTRE/DRE/CRT** (low temperature response element, drought responsive element, or c-repeat) domain in their promoter that is recognised by **C-repeat/dehydration-responsive element binding factors /Dehydration-responsive element-binding1 (CBF/DREB1)** (Svensson *et al.*, 2002; Welling *et al.*, 2004). CBFs can induce the expression of a set of cold-responsive genes to increase plant cold tolerance and is mainly induced by **CBF EXPRESSION1 (ICE1)**; Thomashow, 2010; [Figure 2.17](#)). Expression of a tea (*Camellia sinensis* (L.) O. Kuntze) *CBF1* (*CsCBF1*) was induced immediately and significantly by low temperature (4°C) and not by normal warm growth temperature (20°C; Wang *et al.*, 2012). It has been shown that *CBF* genes play a role in dormancy onset and release by the ectopic expression of a peach (*Prunus persica* L. Batsch) *CBF* (*PpCBF1*) in apple (*Malus x domestica* Borkh.) showing a growth inhibition, induction of early bud set and leaf senescence and a delay of bud break in spring (Wisniewski *et al.*, 2015). At least three *CBF/DREB1* homologues have been identified in sweet cherry, and the conservation of function has been confirmed by the overexpression of one of the sweet cherry *CBF/DREB1* genes in Arabidopsis (Kitashiba *et al.*, 2003, 2004). In addition to the activation of *CBF/DREB1* genes by low temperatures, several **cold-regulated (COR)** genes have been characterised in Arabidopsis plants overexpressing *CBF* genes (Jaglo-Ottosen *et al.*, 1998; [Figure 2.17](#)). The expression of sweet and sour cherry homologues of the Arabidopsis *COR47* gene increases during cold acclimation (Zalunskaitė *et al.*, 2008). These results suggest that the process of cold acclimation is well conserved among plant species. This acclimation process, however, is countered by **High expression of osmotically responsive genes1 protein (HOS1)**, a RING finger E3 ligase that mediates ubiquitination and degradation of ICE1 (Dong *et al.*, 2006; [Figure 2.17](#)). In trifoliate orange (*Poncirus trifoliata* L), *PtrHOS1* expression decreases after a cold treatment but also following an ABA treatment (Liu *et al.*, 2010) suggesting a crosstalk regulation by phytohormones and temperature. Additionally, it was observed that some peach *DAM* genes have CRT/DREB response elements in their promoters, one apple *DAM* gene promoter has a CRT-like element (Wisniewski *et al.*, 2011) and same observations in leafy spurge *DAM* genes (Horvath *et al.*, 2010), suggesting that cold resistance and dormancy pathways are intertwined.

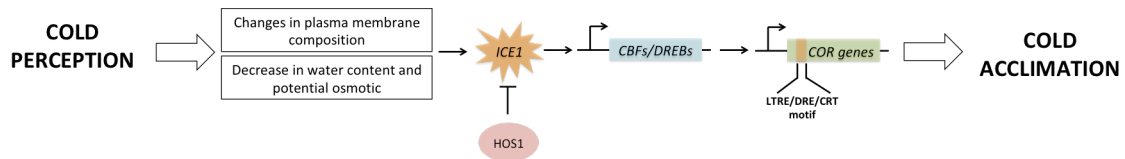


Figure 2.17 | Main changes in plants in response to cold temperature that lead to cold acclimation

2.5.5 KEY POINTS TO REMEMBER

- Dormancy in perennials is controlled at different levels such as **transcriptomic, epigenetic and hormones**.
- A positive correlation has been established between **DAM (DORMANCY ASSOCIATED MADS BOX)** genes and the bud dormancy status, which are belonging to the same family as FLC and SVP in Arabidopsis.
 - Up-regulation of *DAM*-related genes in dormant buds.
 - Down regulation during dormancy release and before bud break.
- *DAM* gene expressions are regulated by repressive and active histone marks (H3K27me3 and H3K4me3, respectively).
- Other epigenetic mechanism appeared to be involved in the regulation of dormancy: **dormancy onset and release is regulated by DNA methylation**.
- **Hormones are key features to control dormancy in perennials** as found in dormancy in seed.
- **Abscisic acid** would function as a signal in response to autumn short days and decreasing temperatures to induce dormancy onset.
- **Gibberellic acid** would maintain growth before the induction of dormancy and promoting growth during ecodormancy.
- **Cold acclimation and hardiness pathways and dormancy pathways are intertwined**.
- Other important pathways have been founded to regulate dormancy: **Reactive oxygen species, cell-to-cell communication, carbohydrates and photoperiod**.

3. Objectives

Predictive climatic models confirm an acceleration of global warming with an increase of temperature of 2°C to 4°C expected by 2050 (IPCC, 2013). In this context, several questions remain open concerning plant adaptation to climate change. This is particularly true for perennial plants as flowering occurs during the subsequent season, exposing reproductive structures to high temperature variations, and even more for temperate fruit trees, given the duration from fruit tree selection to cultivation and commercialisation. It has been shown that an increase in temperature will lead to disorders in phenology, increasing the risk of frost damage and low bud burst rate. These phenological changes have a direct impact on the production of fruit crops and result in a large economic loss, but global change consequences do not appear to have been sufficiently taken into account in the fruit tree industry, despite the high vulnerability of the sector. **The economic and social challenge is to develop new agronomic practices adapted to climate change in order to maintain and expand the fruit production.**

As a consequence, plant responses to ambient temperature should be a priority target for breeding strategies. Cultivars with low chilling requirements will be needed in regions where in the future warm winters may not allow the full release of dormancy. Similarly, controlling how fruit trees respond to warm temperatures will be critical to prevent early frost damage. Taking these criteria into account, another crucial aspect is therefore to better understand the interactions between cold and warm temperatures during dormancy and how they affect contrasted cultivars. In addition, precise phenological modelling of climate change is needed to predict regions that will become unfavourable or favourable for growth and production. However, molecular and physiological markers of dormancy and dormancy release, which are required to set up these models, are currently lacking. In this context, my project aimed to provide knowledge and tools to assist in the adaptation of sweet cherry cultivars for future climatic conditions.

The first objective of this thesis is to better understand molecular processes involved in dormancy and bud break. To do this, we used a combination of complementary 'omics' approaches: hormones quantification by UHPLC-MS/MS (collaboration with the Roullier Group), transcriptomics and epigenomics (collaboration with Philip A. Wigge, University of Cambridge, UK). This study was conducted on sweet cherry (*Prunus avium* L.). This species has a particularly well-organised architecture, with pre-determined floral and vegetative buds,

allowing the molecular and physiological mechanisms to be precisely tracked in buds from the initiation of flowers to bud break and flowering. Moreover sweet cherry trees are highly sensitive to temperature changes since its dormancy onset and release are triggered predominantly by low temperatures rather than by short photoperiods, as is the case for other perennials. The epigenome and transcriptome analyses in response to seasonal changes were investigated in sweet cherry floral buds using RNA-seq and ChIP-seq (Chapters 5, 6 and 7). Buds for several cherry varieties with different chilling and heat requirements were harvested over several months spanning the entire period of bud dormancy until bud break. The analyses allowed us to identify key genes whose expression is altered during the course of dormancy and to determine whether those genes might potentially be regulated by epigenetic mechanisms. Hormonal pathways involved in dormancy regulation were further investigated to better characterise their role in contrasted cultivars (Chapter 8). These studies represent an extremely valuable source of information for genetic studies and to create more accurate predictive models, which will be powerful tools to assist the breeding strategies.

The creation of a new cultivar is a long process lasting up to twenty years, so it becomes urgent to find short- to mid-term solutions to protect the endangered cherry production due to climate change. One such strategy is the application of biostimulants to counteract insufficient chilling requirements arising as a result of mild winters. Therefore, **the second objective of this thesis is to identify biostimulants of dormancy release in sweet cherry**. This was done in collaboration with the Roullier Group by testing the impact of several biostimulants on dormancy release. The effect of one of the best candidates having an impact on dormancy release was further analysed using biochemical and molecular approaches to better understand its effect on dormancy progression (Chapter 9).

The two thesis objectives are complementary since the study of marker genes involved in dormancy to flowering will provide useful knowledge to create decision-making tools as well as predictive models for growers who need to know the specific timing to apply dormancy release products. On the other hand, the characterisation of new products impacting bud dormancy will help to distinguish main biological pathways involved in bud break, thus gaining an understanding of the regulation of this critical step.

4. Materials and methods

Test of products on cut branches and trees, estimation of dormancy release for several cultivars were carried out at INRA Bordeaux-Aquitaine, Grande-Ferrade (Fr). Phytohormone and sulphur metabolite quantification were performed at C.M.I, Roullier Group, St Malo (Fr) and all molecular assays were carried out at the Sainsbury Laboratory, Cambridge (UK).

4.1 Plant materials and methods

4.1.1 Cultivars studied

All cherry cultivars studied come from the National Collection or from the Cherry INRA Collection preserved in the Biological Resources Centre (CRB) of *Prunus* led by the Fruit Experimental Unit (UEA).

Four sweet cherry cultivars displaying different flowering time were used for molecular studies such as RNA-seq, RT-qPCR and ChIP-seq (Table 4.1 and Figure 4.1). ‘Cristobalina’ is a Spanish low-chilling cultivar with an early flowering time. ‘Garnet®’ is an American medium-chilling cultivar with an early flowering time. ‘Bigarreau Hatif Burlat’ or simply called ‘Burlat’ is a French medium-chilling cultivar with a short fruiting period. This is an old cultivar used both in France and in other part of the world. ‘Regina’ is a German high-chilling cultivar with a late flowering time.

On top of these cultivars, two other sweet cherry cultivars displaying different flowering time (Table 4.1 and Figure 4.1) and an apple cultivar were used to test the effect of chemicals on bud break and dormancy release. ‘Fertard’ is a French high-chilling sweet cherry cultivar, ‘Folfer’ is a French low-chilling sweet cherry cultivar and ‘Choupette’ is a French apple cultivar (*Malus x domestica* Borkh.), several trees of each cultivar are present in our orchard.

Cultivar	‘Burlat’	‘Cristobalina’	‘Folfer’	‘Garnet’	‘Regina’	‘Fertard’
Origin	France	Spain	France	USA	Germany	France
Blooming time	intermediate	very early	very early	early	very late	very late
Ripening time	very early	very early	7-13 days after ‘Burlat’	15 days after ‘Burlat’	28-35 days after ‘Burlat’	31-39 days after ‘Burlat’
Productivity	very good	medium to good	very good	good	very good	medium to good

Table 4.1 | Characteristics of cultivars studied (part 1)

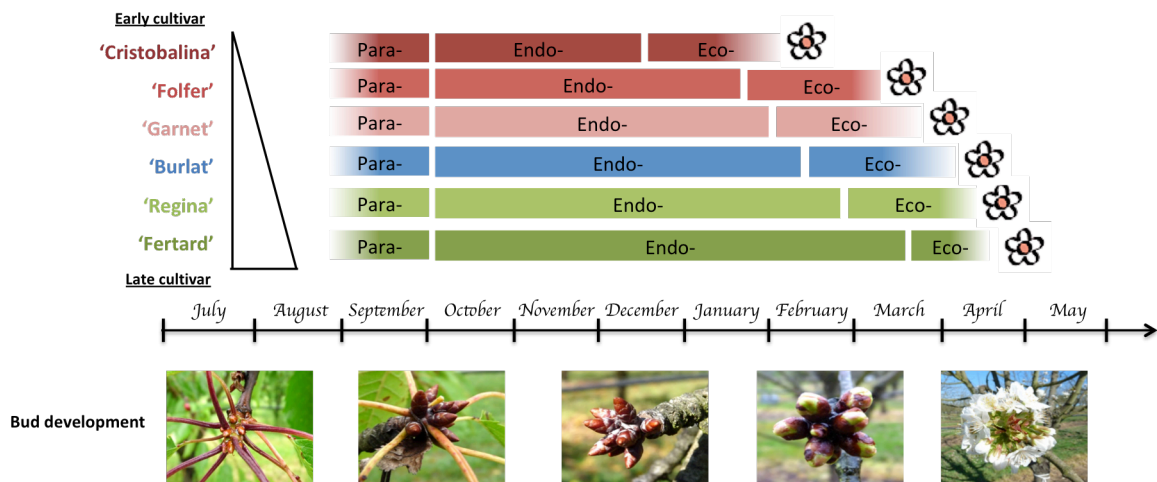


Figure 4.1 | Characteristics of varieties studied (part 2)

4.1.2 Natural growth conditions (orchards)

Cherry trees are grown in orchards located at the Fruit Experimental Unit of INRA in Toulence (48° 51' 46" N, 2° 17' 15" E) and in Bourran (44° 19' 56" N, 0° 24' 47" E) in Nouvelle-Aquitaine (South West of France) under standard agricultural practices. Trees are planted with 5 meters between trees and 6 meters between rows.

In Bourran, the soil characteristics are 1/3 silt, 1/3 clay and 1/3 sand. The climate is oceanic and also continental, with cold winters and 700 millimetres of rainfall per year. Summers are dry so the soil requires a spay irrigation.

In Toulence, the soil is characterised by the presence of fertile alluvium less sensitive to drought. The climate is oceanic with 850 millimetres of rainfall per year. The spay irrigation is used when needed.

In both places, orchards are supplied with sensors that record the temperature every two hours.

4.1.3 Measurements of bud break and estimation of the dormancy release date

Measurements for the dormancy stages were performed on randomly chosen cut branches from 'Burlat' tree in Toulence. The branches were harvested every two weeks from October 1st 2014 to January 27th 2015. Branches from 'Cristobalina', 'Garnet', 'Burlat' and 'Regina' trees in Bourran were harvested every two weeks from November 16th 2015 to April 1st 2016. Branches were incubated in water pots placed in a growth chamber (25°C, 16h light/ 8h dark, 60-70% humidity). The water was replaced every 3-4 days. When the first flower bud reached the BBCH stage 53 (Meier *et al.*, 1994; Fadón *et al.*, 2015; Figure 4.2

and Appendix Figure 1 and 2), phenological observations were carried out on flower buds every two days. The date of dormancy release was evaluated when the percentage of bud break reached 50% of buds at the BBCH stage 53. The data are represented in percentage of buds at the BBCH stage 53 for three branches over the course of time.

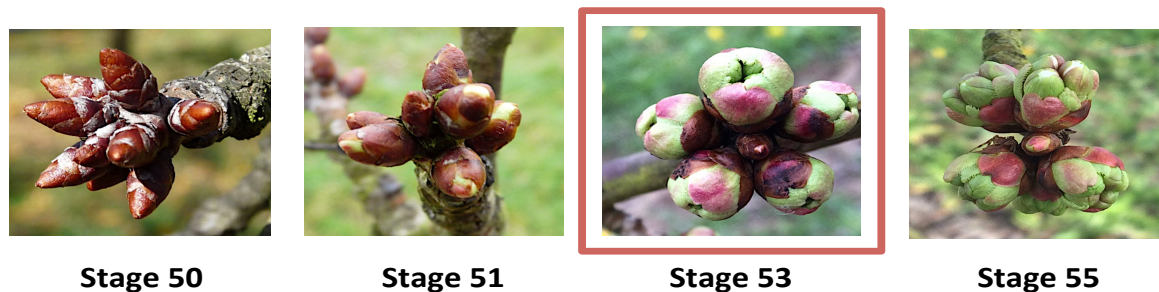


Figure 4.2 | Dormancy release phases of sweet cherry flower buds according to the BBCH scale
The principal stages of dormancy release in flower buds, Stage 50: inflorescence bud closed; Stage 51: inflorescence buds swelling; Stage 53: bud burst and Stage 55: single flower buds visible (Meier *et al.*, 1994; Fadón *et al.*, 2015).

4.1.4 Epigenetic drugs and Roullier products studied

Chemicals tested were chosen for their potential effect on dormancy. For confidential reasons, the composition of Roullier products remained unknown at all stages of the experimental trial.

Different groups of epigenetic drugs were also tested on floral buds in order to explore the role of epigenetic mechanisms involved in dormancy and dormancy release (Appendix Table 7). Nicotinamide, trichostatin A, vorinostat (SAHA) and sodium butyrate are group members of histone deacetylase (HADC) inhibitors. Anacardic acid and curcumin are group members of histone acetyltransferase (HAT) inhibitors. 5-azacytidine is a group member of DNA methyltransferase (DNMT) inhibitor. These chemicals were tested at different concentrations during different winter periods only on cut branches in a confined structure, as they are not certified to be tested outside.

Five Roullier products (OKEN, UDRA, SPEFLO, BASIC and FLOU) were tested at different concentrations during winter 2015/2016. Following this first trial, three products (OKEN, UDRA and SPEFLO) were chosen for further tests because of their impact on dormancy release. Three chosen products and 2 new products (LASAT and ADD-01) were tested on cut branches at different concentrations during winter 2016/2017. The modality “two applications of product” with a one-week interval between applications was added to observe the response to two doses of product. Finally during winter 2017/2018, different

formulations of OKEN were tested (OKEN, OKEN batch A, OKEN batch B, OKEN batch C and OKEN batch D) with only one concentration but two applications.

For all assays, a water control was used using distilled water instead of products. Dormex® was also used as a positive control. This product is composed of hydrogen cyanamide, which is known to accelerate and synchronise dormancy release in several stone fruit trees. Dormex® was tested on cut branches in a confined structure only, as this product is no longer certified to be tested outside in France and in other European countries.

4.1.5 Application of products on cut branches in controlled conditions (growth chamber)

Products from the Roullier Group and epigenetic drugs were tested on cut branches from 'Fertard' cherry cultivar during different winter periods. The application concentrations for epigenetic drugs were chosen in function of the plant science literature.

Firstly, five branches per modality were randomly harvested from trees in an orchard located in Toulence when trees had reached three quarter of chilling requirements, based on the model of Fishman *et al.*, (1987). All products and controls (water and Dormex®) were freshly prepared to the desired concentrations; Table 4.2) in 0.5% of surfactant ("Calanque", Action Pin, Castets, France) to ensure the penetration of active molecules through the bud scales. Roullier products were sprayed and epigenetic drugs were applied using a pipette under a fume-hood and branches were left under the fume-hood several minutes to allow them to dry. Branches were then transferred in the growth chamber (25°C, 16h light/ 8h dark, 60-70% humidity; Figure 4.3) in pots containing water. The water was replaced every 3-4 days. Bud break measurements were performed on flower buds as mentioned above in "4.1.3 Measurements of bud break and estimation of the dormancy release date".

			2015/2016	2016/2017	2017-2018
Epigenetic drugs	HDAC inhibitor	Nicotinamide (mM)	[5], [10]	/	[10]
		Trichostatin A (μM)	[5], [10]	/	/
		Vorinostat (μM)	/	[1], [5]	/
		Butyrate(mM)	/	[1], [10]	/
	HAT inhibitor	Anacardic acid (μM)	/	[1], [10]	/
		Curcumin(μM)	/	[5], [10]	[10]
	DNMT inhibitor	5-azacytidine (μM)	/	[50], [100]	/
Roullier products		FLOU (%)	[0,05], [0,1], [0,2]	/	/
		OKEN (%)	[0,137], [0,275], [0,550]	[0,275], [0,550], [0,550] x 2, [1,1]	[0,550] x 2
		BASIC (%)	[0,05], [0,1], [0,2]	/	/
		UDRA (%)	[0,115], [0,230], [0,460]	[0,115], [0,230], [0,230] x 2, [0,460]	/
		SPEFLO (%)	[0,2], [0,4], [0,8]	[0,2], [0,4], [0,4] x 2, [0,8]	/
		LASAT (%)	/	[0,2], [0,4], [0,4] x 2, [0,8]	/
		ADD-01 (%)	/	[0,01], [0,05], [0,25], [0,25]x2	/
		OKEN (%)	[0,137], [0,275], [0,550]	[0,275], [0,550], [0,550] x 2, [1,1]	[0,550] x 2
		OKEN batch A (%)	/	/	[0,550] x 2
		OKEN batch B (%)	/	/	[0,550] x 2
		OKEN batch C (%)	/	/	[0,550] x 2
	OKEN batch D (%)	/	/	[0,550] x 2	
Controls	Control	Water	yes	yes	yes
	Positive control	Dormex® (%)	[2]	[2]	[2]

Table 4.2 | List of chemicals used in this study and their concentrations

HDAC: histone deacetylase, HAT: histone acetyltransferase and DNMT: DNA methyltransferase

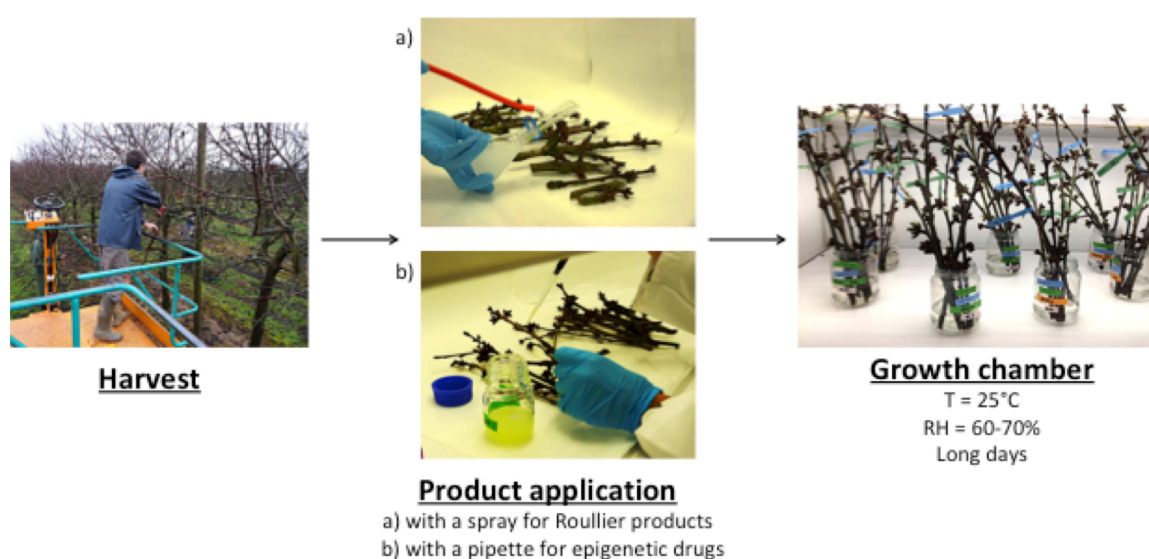


Figure 4.3 | Workflow for the test of chemicals on cut branches

Firstly, branches were randomly harvested from 'Fertard' trees. a) Roullier products were sprayed and b) epigenetic drugs were applied using a pipette under a fume-hood. Then, branches were placed in water trays in the growth chamber (25°C, 16h light/ 8h dark, 60-70% humidity).

4.1.6 Application of Roullier products on trees in natural conditions (orchards)

a) Winter 2016/2017

After a series of test in controlled condition (cut branch assays), Roullier products that have shown the best results on dormancy release were selected (OKEN and SPEFLO) as well as their optimum concentration (0.550% for OKEN and 0.4% for SPEFLO). However, we also wanted to confirm whether the effects seen in controlled conditions would be similar in natural conditions (orchards).

During winter 2016/2017, OKEN and SPEFLO were sprayed on ‘Fertard’ trees (high-chilling cultivar) in mid-January when trees had reached three quarter of chilling requirements, based on the model of Fishman et al., (1987). This is an optimal period for product application on fruit trees commonly used by growers. The products were sprayed on the overall surface of the trees using a manual crop sprayer. All products and the water control were freshly prepared to the desired concentrations (Table 4.2) in 0.5% of surfactant (“Calanque”, Action Pin, Castets, France) with two trees per modality. Two modalities per product were chosen:

1) One application of the double dose of the optimum concentration (1.1% for OKEN and 0.8% for SPEFLO; Table 4.3).

2) Two applications of the optimum concentration with a one-week interval between applications (2 x 0.550% for OKEN and 2 x 0.4% for SPEFLO; Table 4.3), weather permitting (no rain, no wind).

Flower buds were harvested from trees every week and directly flash frozen in liquid nitrogen for molecular studies. Branches were also harvested every week and incubated in the growth chamber in order to estimate the dormancy release date for different treatments. This estimation was done with the measurements of bud break on flower buds as mentioned before in “4.1.3 Measurements of bud break and estimation of the dormancy release date”.

<u>‘Fertard’</u>	OKEN (%)	SPEFLO (%)	Water
2016/2017	[1,1]	[0,8]	
	[0,550] x 2	[0,4] x 2	yes
Number of trees	2	2	
	2	2	2

Table 4.3 | Application concentrations of Roullier products on ‘Fertard’ trees during winter 2016/2017

The abundance of flowers was estimated in spring by Lydie Fouilhaux, field technician INRA-A3C team. Unfortunately, the measurement of fruit yield was impossible due to the presence of the harmful Asian fruit flies (*Drosophila suzukii*) that destroyed most of the cherry production (Figure 4.4).

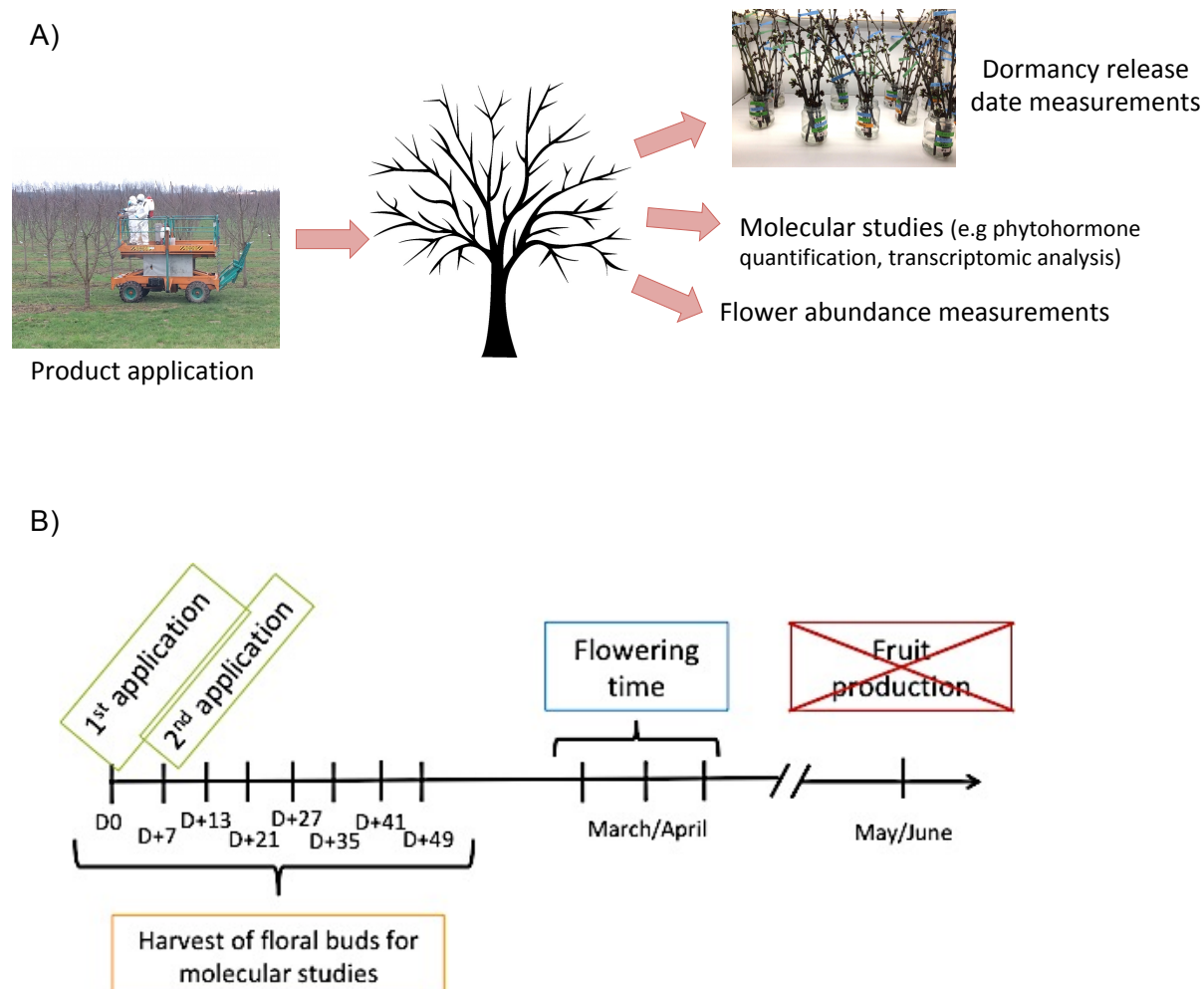


Figure 4.4 | Workflow for the test of Roullier products on trees

After product applications on trees, a) different measurements were carried out in order to observe the effects of products on bud break and dormancy release. b) The workflow schedule for the applications, harvests, flowering time and fruit production measurements.

b) Winter 2017/2018

During winter 2017/2018, different formulations of OKEN (OKEN, OKEN batch A and OKEN batch B) and a water control were tested on two sweet cherry cultivars displaying different flowering time ('Fertard' and 'Folfer') and on an apple cultivar ('Choupette'). The use of different cherry cultivars and different species allow defining the action spectrum of products.

Different formulations of OKEN (OKEN, OKEN batch A and OKEN batch B) and a water control were sprayed on 'Folfer' trees in beginning January and on 'Fertard' trees in mid-January when three quarter of chilling requirements were not reached in order to assess the potential toxicity of OKEN when the product is apply earlier than it should be. Additionally, OKEN was also sprayed on other 'Folfer' trees at end January and on other 'Fertard' trees at beginning February when trees have reached three quarter of chilling requirements to observe the product effects on dormancy release in low-chilling cultivar (Figure 4.5).

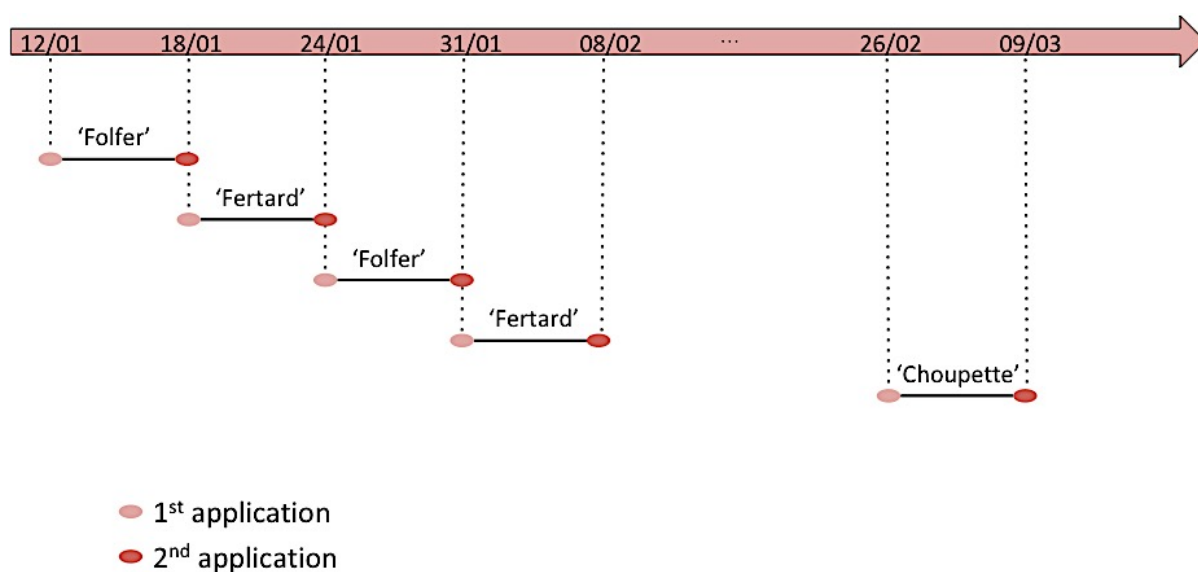


Figure 4.5 | Application dates of Roullier products during winter 2017/2017 in different species
 'Folfer' is a low-chilling sweet cherry cultivar, 'Fertard' is a high-chilling sweet cherry cultivar and 'Choupette' an apple cultivar.

In all cases, the products were sprayed on the overall surface of the trees using an electric crop sprayer. All products and the water control were freshly prepared to the desired concentration (Table 4.4 A and B) in 0.5% of surfactant ("Calanque", Action Pin, Castets, France). The number of trees was limited so the allocation of trees per product was decided by the agrotech company. The modality "two applications of the optimum concentration" was chosen. Flower buds were harvested from trees every week or two weeks and directly flash frozen in liquid nitrogen for molecular studies. Branches were also harvested every week or two weeks and incubated in the growth chamber in order to estimate the dormancy release date for different treatments. This estimation was done with the measurements of bud break on flower buds as mentioned before in "4.1.3 Measurements of bud break and estimation of the dormancy release date". The abundance of flowers was estimated in spring by Lydie Fouilhaux, field technician in INRA-A3C team (Figure 4.4).

OKEN and a water control were sprayed on ‘Choupette’ trees, an apple cultivar, at end February to observe the effect of products on another fruit tree specie that have also a dormancy period led by temperature. The products were sprayed on the overall surface of the trees using an electric crop sprayer. All products and the water control were freshly prepared to the desired concentrations (Table 4.4 C) in 0.5% of surfactant (“Calanque”, Action Pin, Castets, France). The flowering date was estimated in spring by François Gazel, field technician in INRA-UEA.

A)

<u>‘Folfer’</u>	OKEN (%)	OKEN lot A (%)	OKEN lot B (%)	OKEN (%) 2 nd date	Water
2017/2018 (12-18 January)	[0,550] x 2	[0,550] x 2	[0,550] x 2	[0,550] x 2	yes
Number of trees	2	2	2	2	2

B)

<u>‘Fertard’</u>	OKEN (%)	OKEN lot A (%)	OKEN lot B (%)	OKEN (%) 2 nd date	Water
2017/2018 (18-24 January)	[0,550] x 2	[0,550] x 2	[0,550] x 2	[0,550] x 2	yes
Number of trees	2	1	1	2	1

C)

<u>‘Choupette’</u>	OKEN (%)	Water
2017/2018 (26-09 March)	[0,550] x 2	yes
Number of trees	2	2

Table 4.4 | Application concentrations of Roullier products during winter 2017/2018
(A) ‘Folfer’ sweet cherry trees, (B) ‘Fertard’ sweet cherry trees and (C) ‘Choupette’ apple trees.

4.1.7 Material sampling

a) Winter 2014/2015

Flower buds were harvested from ‘Burlat’ trees located in Toulonne every two weeks from 1st October 2014 to 1st March 2015. According to the results from the measurements of bud break and the estimation of the dormancy release date, three time points were chosen

to perform molecular analyses: 21st October 2014 and 5th December 2014 for dormant buds, and 27th January 2015 for non-dormant buds (Table 4.5). Buds were harvested from the same branch, one branch per replicate with three replicates in total. Upon harvesting, buds were flash frozen in liquid nitrogen and stored at -80°C prior to performing ChIP-seq and RNA-seq.

Harvest Date	Cultivar	Dormancy state
21 st October 2014	'Burlat'	D
05 th December 2014	'Burlat'	D
27 th January 2015	'Burlat'	ND

Table 4.5 | Bud harvest dates for 'Burlat' during winter 2014/2015 to perform RNA-seq and ChIP-seq
Dormancy state: Dormant (D) and non-dormant (ND).

b) Winter 2015/2016

Flower buds were harvested from 'Cristobalina', 'Garnet', 'Burlat' and 'Regina' trees located in Bourran every week from 1st July 2015 to 1st April 2016. According to the results from the measurements of bud break and the estimation of the dormancy release date, ten time points spanning the entire period of bud dormancy up to bud break were chosen for molecular analyses (Table 4.6). Buds were harvested from the same branch, from three different trees, one branch per replicate with three replicates in total. Except for 'Regina', where only two trees were present in two copies. Upon harvesting, buds were flash frozen in liquid nitrogen and stored at -80°C prior to performing RNA-seq.

Harvest Date	Cultivars	Dormancy state
07 th July 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	D, D, D, D
04 th August 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	D, D, D, D
01 st September 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	D, D, D, D
06 th October 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	D, D, D, D
03 rd November 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	D, D, D, D
01 st December 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	D, D, D, D
15 th December 2015	'Cristobalina'	ND
29 th December 2015	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	ND, D, D, D
13 th January 2016	'Garnet'	D
26 th January 2016	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	ND, ND, D, D
09 th February 2016	'Burlat'	ND
23 th February 2016	'Cristobalina', 'Garnet', 'Burlat', 'Regina'	ND, ND, ND, ND
08 th March 2016	'Regina'	ND
22 th March 2016	'Garnet', 'Burlat', 'Regina'	ND, ND, ND

Table 4.6 | Bud harvest dates for different sweet cherry cultivars displaying different flowering date during winter 2015/2016 to perform RNA-seq.
Dormancy state: Dormant (D) and non-dormant (ND).

c) Material sampling for molecular tests

Branches were harvested from 'Burlat' trees located in Toulence at different dates and brought in the laboratory at INRA-La Grande-Ferrade to remove scales from flower buds using a scalpel. Quickly, buds without scales were cross-linked with a formaldehyde solution under a vacuum. After cross-linking, buds were rinsed with milliQ water, flash frozen in liquid nitrogen and stored at -80°C. ChIP-qPCR were carried out on material cross-linked before being flash-frozen and pulverised as well as on material flash-frozen and pulverised prior to cross-linking in order to see if the presence or absence of scales may influence ChIP results.

RNA extraction and quantitative real-time PCR were performed on flower buds with and without scales in order to see if the presence or absence of scales may influence the RNA extraction and consequently also gene expression results. Scales were removed from buds after flash-freezing using tweezers. Buds came from 'Garnet' trees during the 2015/2016 sampling period.

4.2 Molecular biology procedures

4.2.1 qRT-PCR reactions

For expression analyses by quantitative PCR, total RNA was extracted from 50-60 mg of frozen and pulverised flower buds using RNeasy Plant Mini kit (Qiagen) with minor modification (1.5% PVP-40 was added in the RLT buffer). Genomic DNA was eliminated by DNase I treatment using the TURBO DNA-free kit (AM1907, Invitrogen) and purified RNA was reverse-transcribed using the Transcriptor First-Strand cDNA Synthesis Kit (04379012001, Roche), according to manufacturer instructions.

Quantitative real-time PCR (qRT-PCR) was carried out using LightCycler 480 DNA SYBR Green I Master mix (04707516001, Roche) with 0.3 µM primers and 2 µl cDNA diluted 1:10 after synthesis. Fluorescence was quantified for 45 PCR cycles with 15 second 94°C denaturation, 30 second 60°C annealing and 30 second 72°C extension (LightCycler 480, Roche). Primers used for qRT-PCR are given in [Appendix Table 8](#).

4.2.2 ChIP-seq and RNA-seq

Chromatin immunoprecipitation-sequencing and RNA-sequencing for complex and low-abundance tree buds

Noémie Vimont^{1,2}, David Schoepfer², François Roudier^{3,4}, Elisabeth Dirlwanger¹, Philip A. Wigge², Bénédicte Wenden¹ & Sandra Cortijo²

¹UMR 1332 BFP, INRA, Univ. Bordeaux, F-33140 Villenave d'Ornon, France

²The Sainsbury Laboratory, University of Cambridge, Cambridge, CB2 1LR, United Kingdom

³Institut de Biologie de l'Ecole Normale Supérieure, Centre National de la Recherche Scientifique (CNRS) UMR8197, Institut National de la Santé et de la Recherche Médicale (INSERM) U1024, Paris, France

⁴ Present address: Laboratoire Reproduction et Développement des Plantes, Univ Lyon, ENS de Lyon, UCB Lyon 1, CNRS, INRA, F-69342, Lyon, France

Key words: Tree buds, ChIP-seq/ Chromatin immunoprecipitation-sequencing, RNA-seq/RNA-sequencing, *Prunus avium* L., *Prunus persica* (L.) Batsch, *Malus x domestica* Borkh.

BACKGROUND

Chromatin immunoprecipitation-sequencing (ChIP-seq) is a robust technique to study interactions between proteins, such as histones or transcription factors, and DNA. This technique combined with RNA-sequencing (RNA-seq) is a powerful tool to better understand biological processes in eukaryotes.

RESULTS

We developed a combined ChIP-seq and RNA-seq protocol for tree buds (*Prunus avium* L., *Prunus persica* (L.) Batsch, *Malus x domestica* Borkh.) that has also been successfully tested in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Tree buds contain phenolic compounds that negatively interfere with ChIP and RNA extraction. In addition to solving this problem, our protocol is optimised to work on small amounts of material. In this protocol, samples for ChIP-seq are cross-linked after flash freezing, making it possible to work on trees growing in the field and to perform ChIP-seq and RNA-seq on the same starting material, differentiating it from previously published protocols.

CONCLUSIONS

This protocol will allow analysis of chromatin and transcriptomic dynamics in tree buds, notably during its development and response to the environment (Figure 4.6).

Article in revision, all the protocol is available on:

<https://www.biorxiv.org/content/early/2018/05/30/334474>

The following description is the method.

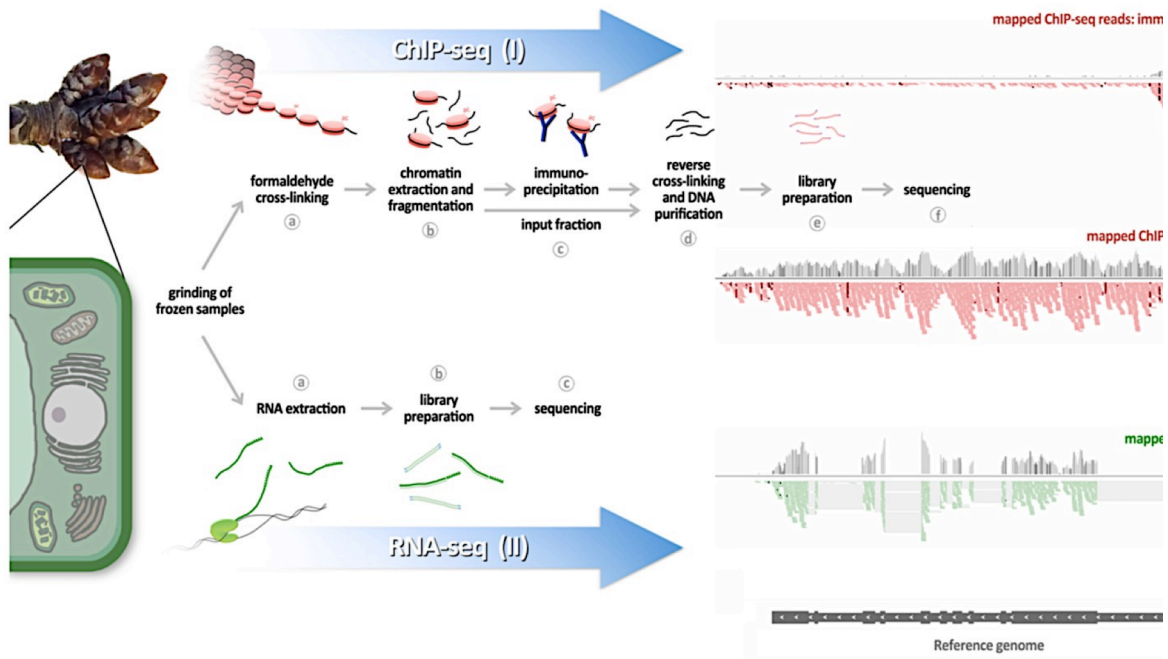


Figure 4.6 | Workflow ChIP-seq and RNA-seq

Outline of the two main modules of the protocol: (I) ChIP-seq (top) and (II) RNA-seq (bottom).

Each module starts with the same biological material (ground frozen material).

(I) (a) The ChIP-seq module starts with a cross-linking step on frozen powder to stabilise interactions between DNA and proteins. (b) The chromatin is extracted using different buffers and then fragmented by sonication or MNase digestion. (c) Proteins of interest, among the protein/DNA complexes, are immunoprecipitated using specific antibodies coupled to magnetic beads. An aliquot of chromatin is set aside as an input fraction, later used for normalisation. (d) After different wash steps, a reverse cross-linking step is performed, and the DNA is isolated using SPRI beads. (e) The purified DNA is used in library preparation, and (f) is then sequenced.

(II) (a) The RNA-seq module starts with RNA extraction from the frozen powder. (b) This DNase treated RNA is then used in library preparation, and (c) sequenced.

MATERIALS AND METHODS

MATERIAL SAMPLING SECTION

Harvest tree buds in 2 ml tubes with screw cap and immediately flash-freeze in liquid nitrogen. There is no need to remove the scales after harvesting. The biological material can be conserved at -80°C up to twelve months before proceeding with ChIP-seq (six months if the tissues are ground into powder). Grind the tissues to a fine powder using mortars and pestles pre-chilled with liquid nitrogen. Add liquid nitrogen several times while grinding to facilitate cell lysis and to ensure that the material remains completely frozen to prevent degradation of tissues.

For the cross-linking and chromatin extraction, weigh out 300 to 500 mg of powder (200 to 400 mg if scales have been removed from the buds) in a 50 ml Falcon tube pre-chilled with liquid nitrogen. The same amount of powder should be used for all samples to allow a direct comparison of results. Then proceed to “ChIP and library preparation section”.

For RNA extraction, weigh out 50-70 mg of powder (20-40 mg without scales) in a 2 ml tubes (screw cap) pre-chilled with liquid nitrogen. Then proceed to “RNA extraction and

library preparation section". Due to the small amount of starting material, it is necessary to keep the tubes in liquid nitrogen to prevent any degradation.

ChIP AND LIBRARY PREPARATION SECTION

Cross-linking and chromatin extraction: Timing 2-3 hours

Add 25 ml of ice-cold Extraction buffer 1 [0.4 M sucrose, 10 mM HEPES pH 7.5, 10 mM MgCl₂, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 % PVP-40 (polyvinylpyrrolidone), 1 tablet of complete protease inhibitor EDTA free for 50 ml of buffer from Sigma cat# 11836170001] to the powder. For each buffer, the protease inhibitor (PMSF), the tablet of complete protease inhibitor and β-mercaptoethanol should be added directly before using the buffer. When extracting chromatin in other biological systems such as *Arabidopsis thaliana* or *Saccharomyces cerevisiae*, the PVP-40 in Extraction buffer 1 may optionally be removed.

Immediately add 675 μl of 37% formaldehyde solution (1% final concentration) and invert the tube several times to resuspend the powder. Cross-link the samples by incubating at room temperature for 10 minutes and then quench the formaldehyde by adding 1.926 ml 2 M of fresh glycine solution (0.15 M final concentration). Invert the tube several times and incubate at room temperature for 5 minutes. Filter the homogenate through Miracloth (Millipore cat# 475855) in a funnel and collect in a clean 50 ml Falcon tube placed on ice. Repeat the filtration step once more. Centrifuge the filtrate at 3,200 × g for 20 minutes at 4 °C. Discard the supernatant by inverting the tube, being careful not to disturb the pellet. Gently resuspend the pellet in 1ml of Extraction buffer 2 [0.24 M sucrose, 10 mM HEPES pH 7.5, 10 mM MgCl₂, 1 % Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1 tablet protease inhibitor EDTA free for 50 ml of solution], without creating bubbles, and transfer the solution to a clean 1.5 ml tube. Centrifuge at 13,500 × g for 10 minutes at 4°C. Carefully remove the supernatant by pipetting. If the pellet is still green, repeat the resuspension in 1 ml of Extraction buffer 2 and centrifugation at 13,500 × g for 10 minutes at 4°C. In a new 1.5 ml tube, add 300 μl of Extraction buffer 3 [1.7 M sucrose, 10 mM HEPES pH 7.5, 2 mM MgCl₂, 0.15 % Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1 mini-tablet protease inhibitor EDTA free for 50 ml of solution]. Slowly resuspend the pellet in 300 μl of Extraction buffer 3 to prevent the formation of bubbles. Take the 300 μl solution of resuspended pellet and carefully layer it on top of the 300 μl Extraction buffer 3. Centrifuge at 21,200 × g for 1 hour at 4°C. During this process, nuclei are pelleted through a sucrose cushion to remove cellular contaminants.

From this step, chromatin fragmentation can be performed in two different ways: (A) sonication, to shear the chromatin into 100-500 bp fragments, or (B) MNase (Micrococcal nuclease) digestion, to enrich for mono-nucleosomes (~150-200 bp).

A. Sonication: TIMING 3-4h hours (+ 8 hours of incubation)

i. Chromatin fragmentation

Carefully remove the supernatant with a pipette and resuspend the nuclei pellet in 300 μ l of Sonication buffer [50 mM HEPES pH 7.5, 10 mM EDTA, 1 % SDS, 0.1 % sodium deoxycholate, 1% Triton X-100, 1 mini-tablet protease inhibitor EDTA free for 50 ml of solution]. To break the nuclear membrane and release the chromatin, flash-freeze the tube in liquid nitrogen and then thaw rapidly by warming the tube in your hand. Repeat once more. Centrifuge the tube at 15,800 \times g for 3 minutes at 4°C to pellet debris, and carefully recover the supernatant into a new tube. Complete the tube to 300 μ l with the Sonication buffer. Set aside a 10 μ l aliquot of chromatin in a PCR tube to serve as the non-sonicated control when assessing sonication efficiency by gel electrophoresis and keep on ice. Shear the chromatin into ~300 bp (100–500 bp) fragments by sonication (e.g. using Diagenode Bioruptor Twin-UCD400, sonicate 300 μ l chromatin in 1.5 ml microcentrifuge tubes for 14 to 16 cycles, on High setting, with 30s ON/30s OFF per cycle). The number of cycles of sonication to obtain DNA fragments of around 300 bp should be tested and optimised for different tissues and different concentrations of chromatin. Transfer 40 μ l of sheared chromatin to a PCR tube, which will be used to check the sonication efficiency. The rest of the sonicated chromatin should be stored at -80°C. Sonicated chromatin can be kept frozen at -80 °C for 3 months without significant loss of chromatin quality.

ii. Analysis of sonication efficiency

Complete the sonicated (40 μ l) and non-sonicated (10 μ l) aliquots to 55.5 μ l with TE buffer [10 mM Tris-HCl pH 8, 1 mM EDTA], add 4.5 μ l of 5 M NaCl and incubate in a PCR machine or thermocycler at 65°C for 8 hours to reverse cross-link. Add 2 μ l of 10 mg/ml RNase A (Fisher cat# EN0531) and incubate at 37°C for 30 minutes. Add 2 μ l of 20 mg/ml proteinase K (Fisher cat# EO0491) and incubate at 45°C for 1 hour. During this step, take out the SPRI beads (e.g. AMPure beads; Beckman Coulter, cat# A63880) from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use).

To extract DNA using SPRI beads, vortex the beads until they are well dispersed, add 126 μ l of beads to 60 μ l of sample (2.1 \times ratio) and mix well by pipetting up and down at least 10 times. Incubate 4 minutes at room temperature and then place the tubes on a magnetic rack (96 well; Fisher, cat# AM10027) for 4 minutes to capture the beads. Carefully remove and discard the supernatant without disturbing the beads. Leave the tubes on the magnetic rack and add 200 μ l of freshly prepared 80 % v/v ethanol. Incubate for 30 seconds and discard the supernatant. Repeat the ethanol wash once more and then completely remove all ethanol. Allow the beads to dry for 15-30 minutes, until cracks appear in the bead pellet and no droplets of ethanol are visible. Tubes can alternatively be placed in a fume hood for 10

minutes to accelerate drying. The beads must be completely free from ethanol as it can interfere with downstream processes. Remove the tubes from the magnetic rack and resuspend the beads in 15 μ l of 10 mM Tris-HCl (pH 8) by pipetting up and down at least 10 times. Incubate for 5 minutes at room temperature and place on the magnetic rack for 4 minutes to capture the beads. Carefully transfer 14 μ l of supernatant containing DNA to a new tube. Add 2.8 μ l of 6 \times Loading dye to 14 μ l of DNA. Separate the DNA by electrophoresis on a 1.5 % agarose gel for at least 1 h at 70 V. The smear should be concentrated between 100–500 bp. If necessary, perform additional sonication cycles. Otherwise, continue directly to the “Immunoprecipitation (IP)” step.

B. MNase digestion: TIMING 4-5 hours (+ 2 \times 8 hours of incubation)

i. DNA quantification prior to MNase digestion

Carefully remove the supernatant with a pipette and resuspend the nuclei pellet in 500 μ l of MNase buffer [20 mM HEPES pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 % NP-40, 3mM CaCl₂, Triton X-100, 1 mini-tablet protease inhibitor EDTA free for 50 ml of solution]. To break the nuclear membrane and release the chromatin, flash-freeze the tube in liquid nitrogen and then thaw rapidly by warming the tube in your hand. Repeat once more. Transfer 40 μ l of chromatin to a PCR tube to quantify DNA prior to MNase digestion and complete to 55.5 μ l with MNase digestion buffer, add 4.5 μ l of 5M NaCl and incubate in a PCR machine or thermocycler at 65°C for 8 hours to reverse cross-link. Keep the rest of the chromatin at –80 °C. Add 2 μ l of 10 mg/ml RNase A and incubate at 37°C for 30 minutes. Add 2 μ l of 20 mg/ml proteinase K and incubate at 45°C for 1 hour. During this step, take out the SPRI beads from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use). Proceed to the DNA extraction using SPRI beads as explained before in the sonication analysis section (ii). Use 1 μ l from each sample to quantify the DNA using a Qubit fluorometer (ThermoFisher Scientific), or a Nanodrop spectrophotometer (Thermo Scientific).

ii. MNase digestion

Adjust all samples to the same concentration according to the quantification results using MNase buffer and to a final volume of 500 μ l. Set aside a 20 μ l aliquot of chromatin in a PCR tube to serve as the non-digested control when assessing MNase efficiency by gel electrophoresis and keep on ice. Incubate chromatin in a ThermoMixer (Eppendorf) for 2-5 minutes at 37°C with shaking at 1,200 rpm, for optimal MNase activity. Add MNase (Fisher cat# 88216) to the chromatin to a final concentration of 0.6 U/ml and incubate 10 minutes in the ThermoMixer at 37°C, 1,200 rpm. Stop the digestion by adding 5 μ l of 0.5 M EDTA pH 8 (5 mM final concentration), invert the tube several times to mix and immediately place on ice

for 5 minutes. The optimal MNase enzyme concentration and incubation time to obtain predominantly mono-nucleosomes should be tested and optimised for different tissues and different concentrations of chromatin. For the optimisation of MNase digestions, we recommend using 1 ml of chromatin and carrying out digestions in 100 μ l aliquots with varying concentrations of MNase (0.2 U/ml to 1 U/ml) and incubation times (5 to 20 minutes). Transfer 50 μ l of digested chromatin to a PCR tube, which will be used to check the MNase efficiency. The rest of the digested chromatin should be stored at -80°C and can be kept at -80 °C for 3 months without significant loss of chromatin quality.

iii. MNase digestion analysis

Complete the digested sample (50 μ l) and non-digested (20 μ l) aliquots to 55.5 μ l with TE buffer, add 4.5 μ l of 5M NaCl and incubate in a PCR machine or thermocycler at 65°C for 8 hours to reverse cross-link. Add 2 μ l of 10 mg/ml RNase A and incubate at 37°C for 30 minutes. Add 2 μ l of 20 mg/ml proteinase K and incubate at 45°C for 1 hour. During this step, take out the SPRI beads from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use). Proceed to the DNA extraction, using SPRI beads as explained before in the sonication analysis section (ii). Add 2.8 μ l of 6 \times Loading dye to 14 μ l of DNA. Separate the DNA by electrophoresis on a 1.5% agarose gel for at least 1 h at 70 V. The most abundant band should be 150-200 bp in size, which corresponds to chromatin in mono-nucleosome form, with a less abundant 300-350 bp band (di-nucleosomes) and a faintly visible ~500 bp band (tri-nucleosomes). For optimum sequencing results, approximately 80% of chromatin should be in mono-nucleosome form. If this is judged not to be the case from the gel, it is not possible to carry out further MNase digestions on the chromatin, as EDTA sequesters calcium ions that are required for MNase activity.

Immunoprecipitation (IP): TIMING 7-8 hours (+ overnight incubation)

Transfer 50 μ l of protein A- and/or protein G-coupled magnetic beads (Invitrogen cat# 10-002D and cat# 10-004D, respectively) per IP to a 2 ml tube. Wash the beads with 1 ml of Binding buffer [0.5% (wt/vol) BSA, 0.5% (vol/vol) Tween-20 in PBS (without Ca²⁺, Mg²⁺)] during 5 minutes at 4°C on a rotating wheel (low speed, around one rotation every 5-6 seconds). Place the tubes on a magnetic rack (Thermo Fisher cat# 12321D) until the liquid is clear and remove the supernatant. Repeat three times. After the washes, resuspend the beads in 250 μ l of Binding buffer. Add 5 μ l of antibody per IP to the beads. In our study, we used anti-trimethyl-histone 3 Lys 27 antibody (Millipore cat# 07-449) and anti-trimethyl-histone 3 Lys 4 antibody (Millipore cat#17-614,). Incubate 4 hours on a rotating wheel at 4°C (low speed). During this incubation time, centrifuge the sonicated or digested chromatin at

15,800 × g for 5 minutes at 4°C to pellet debris, and carefully recover supernatant into a new tube. Transfer 100 µl of sonicated chromatin to a new 2 ml tube for one IP, add 900 µl of Binding buffer and keep on ice. Or transfer 200 µl of MNase-digested chromatin to a new 2 ml tube for one IP, add 800 µl of Binding buffer and keep on ice. Transfer 20 µl of sonicated or MNase-digested chromatin as an input fraction (no immunoprecipitation) in a PCR tube and store at -80°C. The rest of the chromatin can be used for another IP or stored at -80°C. After completion of the incubation of protein A/G beads with the antibody, place the tubes containing the antibody-bead complexes on a magnetic rack, remove the supernatant and wash the beads with 1 ml of Binding buffer during 5 minutes at 4°C on a rotating wheel (low speed). Repeat three times. Resuspend the beads in 50 µl of Binding buffer per IP, then add them to the 1 ml of diluted chromatin. Incubate overnight on a rotating wheel at 4°C (low speed). Briefly centrifuge the tube (<3 seconds) to pull down the liquid in the lid of the tube. Place on a magnetic rack and remove the supernatant. Wash the beads to reduce unspecific interactions by incubating 5 minutes at 4°C on a rotating wheel (low speed) with 1ml of the following buffers and total number of washes: a. 5 washes with Low Salt Wash buffer [150 mM NaCl, 0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8]; b. 2 washes with High Salt Wash buffer [500mM NaCl, 0.1% SDS, 1% triton X-100, 2 mM EDTA, 20mM Tris-HCl pH 8]; c. 2 washes with LiCl Wash buffer [0.25 M LiCl 1% NP-40 (IGEPAL), 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8]; d. 2 washes with TE buffer. After the second wash in TE buffer, resuspend the beads in 100 µl of TE buffer and transfer the beads to a PCR tube. Place the tube on a magnetic rack, remove the TE buffer and resuspend the beads in 60 µl of Elution buffer [10 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 300 mM NaCl, 0.5% SDS].

Reverse cross-linking and Elution by proteinase K treatment: TIMING 2 hours (+ 8 hours of incubation)

Defrost the input fraction on ice (20 µl of sonicated or MNase-digested chromatin). Complete the input fraction to 60 µl with the Elution buffer. Incubate the input fraction and the IP sample (60 µl beads-Elution buffer) at 65°C for 8 hours in a PCR machine or thermocycler to reverse crosslink. Add 2 µl of RNase A (10 mg/ml) and incubate at 37°C for 30 minutes. Add 2 µl of Proteinase K (20 mg/ml) and incubate at 45°C for 1 hour. During this step, take out the SPRI beads from the fridge and allow them to equilibrate at room temperature (at least 30 minutes before use). Place the tubes on a magnetic rack to collect the beads, transfer 60 µl of supernatant from each well to a new PCR tubes (or a new 96 wells-plate).

DNA extraction using SPRI beads and qPCR: TIMING 1 hour

Proceed to the DNA extraction, using SPRI beads as explained before in the sonication analysis section (ii) until just before the elution. Remove the tubes from the magnetic rack and for the elution, resuspend the beads in 50 µl of 10mM Tris-HCl (pH 8.0) by pipetting up and down at least 10 times. Incubate for 5 minutes at room temperature and place on the magnetic rack for 4 minutes to capture the beads. Carefully transfer 49 µl of supernatant containing DNA to a new tube. For qPCR analysis, use 1 µl of DNA per 10 µl reaction, from the IP and input. The percentage of enrichment of DNA in the ChIP fraction relative to the input fraction is calculated according to the formula: $(2^{-Cp\ ChIP} / 2^{-Cp\ input}) \times 100$. Keep the rest of the DNA for sequencing (continue to “ChIP library preparation and size selection section”).

ChIP library preparation and size selection: TIMING 2-3 days

i. ChIP library

For the preparation of sequencing libraries, use the entire volume from the IP and 5–10 ng from the input fraction. We recommend carrying out ChIP-seq library preparation using the TruSeq ChIP Sample Prep Kit (Illumina 48 samples, 12 indexes, Illumina, cat# IP-202-1012) with minor modifications.

- 1- The “Purify Ligation Products” section using the gel electrophoresis is eliminated to minimise DNA loss.
- 2- DNA size selection is carried out after the “Enrich DNA fragments” section. This is a required step to increase the visualisation of nucleosome positioning. Smaller and larger reads might disturb the MNase input profile after analysis.

The Illumina TruSeq ChIP Sample Preparation protocol is available at the following URL:

http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqchip/truseq-chip-sample-prep-guide-15023092-b.pdf

ii. Size selection

Size selection is performed using SPRI beads to remove fragments larger than 500 bp and fragments smaller than 100 bp, the latter being most likely adapters contaminations. This size selection improves the resolution of the ChIP-seq peaks obtained from MNase but is not required when using sonication, except in the case of adapter contaminations. Complete the 15 µl of libraries to 100 µl with Resuspension buffer from the TruSeq ChIP Sample Prep Kit. During this step, allow the SPRI beads to equilibrate at room temperature (at least 30 minutes before use) and vortex the them until they are well dispersed. Add 65 µl of SPRI beads (0.65× ratio; to eliminate > 500 bp fragments). Incubate at room temperature for 5

minutes. Place on a magnetic rack for 5 minutes. Transfer 155 μ l of the supernatant (containing < 500 bp fragments) to a new tube. Add 50 μ l of SPRI beads (1.15 \times ratio; to eliminate fragments < 100 bp). Incubate at room temperature for 5 minutes. Place on a magnetic rack for 5 minutes. Remove and discard the supernatant. Without removing the tube from the magnetic rack, add 200 μ l of freshly prepared 80 % ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove and discard the supernatant. Repeat the ethanol wash once more and then remove all ethanol. Allow the beads to dry for 15-30 minutes, until cracks appear in the bead pellet and no droplets of ethanol are visible. The beads must be completely free from ethanol as the latter can interfere with downstream processes. Remove the tube from the magnetic rack and resuspend the beads with 17.5 μ l of Resuspension buffer. Gently pipette the entire volume up and down at least 10 times to mix. Incubate the tube at room temperature for 2 minutes. Place the tube on a magnetic rack at room temperature for 5 minutes. Transfer 15 μ l of the clear supernatant to a new tube. Check the quality of libraries using a 4200 TapeStation or Bioanalyzer instruments (Agilent) following manufacturer's. If the libraries are contaminated with adapters, repeat again the "size selection" step to remove them, otherwise proceed directly with the "quantification and pool of libraries" section. DNA for sequencing can be frozen at -20°C for 3 months without significant loss of quality.

RNA EXTRACTION AND LIBRARY PREPARATION SECTION

RNA extraction: TIMING 2-5 hours

We recommend for the RNA extraction the use of RNeasy® Plant Mini kit from Qiagen (cat# 74904) for less than 50 samples with the following minor modifications:

- 1- Start from 50-70 mg of buds powder with scales or 30-50 mg of buds without scales. Only remove the tubes from the liquid nitrogen when the RNA Extraction buffer is prepared.
- 2- Add 1.5 % of PVP-40 (polyvinylpyrrolidone) in the RLT buffer to chelate phenolic compounds and thus prevent any interaction. Then add the appropriate volume of β -mercaptoethanol mentioned in the Qiagen protocol.
- 3- Add 750 μ l of RNA Extraction buffer (RLT buffer + PVP-40 + β -mercaptoethanol) instead of 450 μ l if the starting material contains scales to increase the RNA yield.

Alternatively, RNA can be extracted using the MagMAX™-96 Total RNA Isolation Kit from Thermo Fisher (cat# AM1830) for more than 50 samples following manufacturer's instructions. RNA can be frozen at -80°C for 2-3 months without significant loss of quality.

RNA library preparation: TIMING 3-4 days

i. RNA library

We recommend carrying out RNA-seq library preparation using the Truseq Stranded mRNA Library Prep Kit from Illumina (96 samples, 96 indexes, Illumina cat# RS-122-2103). Check the quality of libraries using 4200 TapeStation or Bioanalyzer instruments (Agilent) following manufacturer's instructions. If the libraries are contaminated with adapters, continue with the following "size selection" step to remove them, otherwise proceed directly with the "quantification and pool of libraries" section.

ii. Size selection

Add 20 µl of Resuspension buffer from the Illumina kit to 30 µl of libraries. Allow the SPRI beads to equilibrate at room temperature (at least 30 minutes before use) and vortex them until they are well dispersed. Add 47,5 µl of SPRI beads (0.95 × ratio; to eliminate < 150 bp fragments). Incubate at room temperature for 5 minutes. Place on a magnetic rack for 5 minutes. Remove and discard the supernatant. Without removing the tube from the magnetic rack, add 200 µl of freshly prepared 80 % ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove and discard the supernatant. Repeat the ethanol wash once more and then remove all ethanol. Allow the beads to dry for 15-30 minutes, until cracks appear in the bead pellet and no droplets of ethanol are visible. The beads must be completely free from ethanol, as the latter can interfere with downstream processes. Remove the tube from the magnetic rack and resuspend the beads with 32.5 µl of Resuspension buffer. Gently pipette the entire volume up and down at least 10 times to mix. Incubate the tube at room temperature for 2 minutes. Place the tube on a magnetic rack at room temperature for 5 minutes. Transfer 30 µl of the clear supernatant to a new tube. Check the quality of libraries using a D1000 ScreenTape kit on 4200 TapeStation instrument following manufacturer's instructions or Bioanalyzer instruments. DNA for sequencing can be frozen at -20°C for 3 months without significant loss of quality.

QUANTIFICATION AND POOL OF LIBRARIES SECTION

Quantification of RNA and ChIP libraries:

From this step, the quantification and the pool for RNA and ChIP libraries are the same. However, ChIP-seq libraries on one-hand and RNA-seq libraries on the other should be quantified, pooled and sequenced separately. Libraries are quantified Qubit Fluorometer from Thermo Fisher (DNA high sensitivity). Dilute the DNA high sensitivity dye to 1/200 in the DNA high sensitivity buffer (e.g. for 10 samples: mix 1.990 ml of DNA high sensitivity buffer and 10 µl of DNA high sensitivity dye). Add 198 µl of mix in Qubit tubes (Thermo Fisher cat#

Q32856) and 2 µl of DNA (for standard: 190 µl of mix + 10 µl of standard). Vortex and spin down. Quantification is performed using Qubit fluorometer following manufacturer's instructions.

Pool of libraries: TIMING 1 hour

According to the quantification results, dilute libraries at 10 nM using this calcul to convert from ng/µl to nM: $(\text{concentration} \times 10^6) / (\text{size} \times 617.96 + 36.04)$, where concentration is in ng/µl and size in bp. And pool the libraries using 5 µl of each library. Quantify the pool by Qubit as explained before and dilute the pool to the concentration required by the sequencing facility/company or the sequencer system used.

4.2.3 *in vitro* ChIP

This protocol has been optimised especially for cherry tree buds.

->Cloning

I. Gene sequencing

PavDAM5 and *PavDAM6* genes were amplified from flower buds of 'Burlat' cDNA using a Phusion® High-Fidelity DNA Polymerase (M0530S, NEB) with primers 11025+11026 and 11027+11028 (Appendix Table 9), respectively. The amplicons were isolated by gel electrophoresis, purified using the QIAquick Gel Extraction Kit (28706, Qiagen), concentrated by ethanol precipitation and resuspended in 50 µl of milliQ water. The purified DNA of *PavDAM5* and *PavDAM6* were sequenced by Sanger Sequencing (see Appendix Figure 10 for CDS sequences). *PavFT* and *PavDAM4* genes were previously sequenced by Mathieu Fouché (A3C engineer, see Appendix Figure 10).

II. Construction of *pDBleu::N-FLAGx3* and *pDBleu::C-FLAGx3* vectors

pDBleu::N-FLAGx3 and *pDBleu::C-FLAGx3* vectors, containing the N-terminal FLAG tag present in three copies (N-FLAGx3) and the C-terminal FLAG tag present in three copies (C-FLAGx3), respectively, were constructed using Ligation-Independent Cloning (LIC), as described by Li & Evans (1997). All primers for the *in vitro* ChIP assay are in given in the Appendix Table 9.

The binary vector *pDBleu* (Thermo Scientific) was digested with Eco53kl and HindIII-HF. The linearised vector was isolated by gel electrophoresis, purified using the QIAquick Gel Extraction Kit (28706, Qiagen), concentrated by ethanol precipitation and resuspended in milliQ water to 50ng/µl. The digested vector was digested once more with exoIII (NEB) for 15-30 seconds at 14°C, to create 3' sticky ends, and the digestion was halted by adding 50

µl TE buffer and placing the tube on ice. The DNA was isolated by phenol-chloroform extraction, concentrated by ethanol precipitation and resuspended in 10µl milliQ water. N-FLAGx3 and C-FLAGx3 inserts were amplified by annealing primers 13118+13119 and 13120+13121, respectively, at 60°C for 3 minutes. DNA + N-FLAGx3 inserts on the one hand and DNA + C-FLAGx3 inserts on the other hand were annealed at 60°C for 3 minutes, then transformed into heat shock-competent Escherichia coli DH5α cells using 42°C heat shock for 30 seconds. Bacteria were grown on LB (Luria-Bertani) medium (1% w/v sodium chloride, 1% w/v tryptone, 0.5% w/v yeast extract) supplemented with 1.5% w/v Bacto agar (214010, BD) in 10 cm round Petri plates. Positive clones were selected on kanamycin. Overnight bacterial cultures were grown in 5 ml LB media in 50 ml Falcon tubes, with shaking at 230 rpm (Ecotron, Infors HT). Plasmids were purified from overnight cultures using the QIAprep Spin Miniprep Kit (27106, Qiagen) and verified by Sanger sequencing with primers 7066 and 6161.

III. Construction of pDBleu::FLAGx3::gene and pDBleu::gene::FLAGx3 vectors

pDBleu::FLAGx3::DAM4, *pDBleu::FLAGx3::DAM5*, *pDBleu::FLAGx3::DAM6*,
pDBleu::FLAGx3::FT, *pDBleu::DAM4::FLAGx3* , *pDBleu::DAM5::FLAGx3* ,
pDBleu::DAM6::FLAGx3 and *pDBleu::FT::FLAGx3* vectors were constructed using Ligation-Independent Cloning (LIC).

The coding sequences of *PavDAM4* (0,66kb), *PavDAM5* (0,7kb), *PavDAM6* (0,71kb) and *PavFT* (0,38kb) were amplified from flower buds of 'Burlat' cDNA using a Phusion® High-Fidelity DNA Polymerase (M0530S, NEB) with primers 13138+13139, 13134+13135, 13136+13137 and 13140+13141, respectively, for *pDBleu::FLAGx3::gene* and with primers 13144+13145, 13148+13149, 13150+13151 and 13146+13147, respectively, for *pDBleu::gene::FLAGx3*. *pDBleu::N-FLAGx3* and *pDBleu::C-FLAGx3* vectors were digested with Eco53kl. The inserts and vectors were isolated by gel electrophoresis, purified using the QIAquick Gel Extraction Kit (28706, Qiagen), concentrated by ethanol precipitation and then mixed in an equimolar ratio (100-200ng vector backbone) in a 10 µl volume. The DNA was digested with exoIII (NEB), isolated by phenol-chloroform extraction and concentrated by ethanol precipitation and resuspended in 10 µl deionised water (as previously described).

IV. Transformation in bacteria then in yeast

DNA was annealed at 60°C for 3 minutes and then transformed into heat shock-competent Escherichia coli DH5α cells using 42°C heat shock for 30 seconds. Bacteria were grown on LB medium supplemented with 1.5% w/v Bacto agar (214010, BD) in 10 cm round Petri plates. Positive clones were selected on kanamycin. Overnight bacterial cultures were grown

in 5 ml LB media in 50 ml Falcon tubes, with shaking at 230 rpm (Ecotron, Infors HT). Plasmids were purified from overnight cultures using the QIAprep Spin Miniprep Kit (27106, Qiagen) and verified by Sanger sequencing with primers 7066 and 6161. Plasmids were transformed into *Saccharomyces cerevisiae* YEF473a cells (Bi and Pringle, 1996) using the PEG/LiAc method as outlined by Clontech (2009b) and transformants were selected on synthetic drop-out medium lacking leucine (630310, Clontech).

I. Yeast growth

Yeast strains from overnight culture were cultured in 100 ml of synthetic drop-out medium lacking leucine (630310, Clontech) at 30°C and 230 rpm shaking (Ecotron, Infors HT), from OD₆₀₀ 0.1-0.2 until 0.8-0.9 (Ultrospec 10, Amersham Biosciences). Yeast cells were centrifuged at max speed (5810R, Eppendorf) for 2 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 50 ml of cold milliQ water. The tubes were centrifuged once more at max speed for 2 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in a few millilitres of cold milliQ water and transfer to 2ml screw-cap tubes, with 50ml culture equivalent of cells per tube. Cells were harvested by centrifugation at max speed for 12 seconds (5424R, Eppendorf). Quickly, the supernatant were removed by inverting the tubes abruptly and immediately flash freezing the tubes in liquid nitrogen.

II. Yeast lysis and protein extraction

500 µm silica/zirconium beads (11079110, Cole-Parmer) were added up to the 500 µl mark of the 2 ml tube as well as 500 µl of cold Lysis buffer (50 mM HEPES pH 7.3, 200 mM NaCl, 1% v/v Triton X-100, 1 mM PMSF and EDTA-free protease inhibitor cocktail minitab). Cells were lysed using the Fast Prep-24 machine (MP Biomedicals): 30 seconds lysis at 6.5 m/s, with the pre-cooled 24×2 cryohead and placed on ice. Lid of tubes were pierced with a needle (0.5×16 mm) and the tubes were placed with the lid down into a 15 ml Falcon tube containing a P1000 tip (no filter, point-down, with the 2ml tube on top). Falcon tubes were centrifuged at 2,000 rpm for 30 seconds. The 2 ml tubes containing the beads were removed and discarded and the lysates were transferred to new 2 ml microfuge tubes. Another centrifugation was applied at 15,000 rpm for 15 minutes at 4°C. The supernatants were transferred to new 2 ml tubes.

III. DNase treatment

Tris-HCl pH 7.5, CaCl₂ and MgCl₂ were added to final concentrations of 10 mM, 0.5 mM and 2.5 mM, respectively as well as 5 µl of DNase (Turbo DNase-free kit, Ambion). The

tubes were incubated for 1 hour at room temperature in a ThermoMixer (Eppendorf) at 750 rpm. The reaction was stopped by adding EDTA to a final concentration of 5 mM and tubes were placed on ice. The efficiency of the DNase treatment was verified by PCR with yeast-specific primers.

IV. Purification of proteins

An aliquot of anti-FLAG M2 magnetic beads (M8823, Sigma) was placed on ice for 15-20 minutes to equilibrate to 4°C. 25 µl of beads (per IP) were transferred to a 2 ml tube. 1 ml of Binding/Blocking buffer (0.5% w/v BSA, 0.5% v/v Tween-20 prepared in PBS solution) was added to wash the anti-FLAG M2 magnetic beads and the tubes were inverted several times to resuspend the beads. Tubes were placed on a magnetic rack to collect the beads and removed the supernatant. The wash was repeated twice. After washes, beads were resuspended in 50 µl of Binding/Blocking buffer and kept on ice. The beads were added to the lysate (after DNase treatment) and incubated at 4°C for 4 hours on a rotating wheel at low speed. After incubation, the tubes were placed on a magnetic rack to collect the beads. With the tubes on the magnetic rack, the supernatant of each tubes were removed and beads were washed with 1 ml of wash buffers as indicated below by incubating the beads for 10 minutes at 4°C on the rotating wheel for each wash:

- 2× Low salt wash buffer (150 mM NaCl, 0.1% w/v SDS, 1% v/v Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8).
- 2× High salt wash buffer (500 mM NaCl, 0.1% w/v SDS, 1% v/v Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8).
- 2× LiCl buffer (0.25 M LiCl, 1% v/v NP-40 (IGEPAL), 1% w/v sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8).
- 2× TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

Finally, the beads were resuspended in 100 µl of TE buffer and kept on ice.

V. DNA elution

2 µl of 20 mg/ml proteinase K (AM2546, Thermo Scientific) was added, then the tubes were incubated in a ThermoMixer 800 rpm at 45°C for 1 hour to elute the DNA. The tubes were placed on a magnetic rack to collect the beads and transferred the supernatant of each tube to new 2ml tubes.

VI. DNA extraction

SPRI beads (A63880, Beckman Coulter) were equilibrated at room temperature at least 30 minutes prior to use, then mixed vigorously for 10 seconds to fully resuspend them. 260 µl of

SPRI beads (2.6× ratio) was added to the eluted DNA, mixed thoroughly by pipetting up and down at least 20 times and incubated 4 minutes at room temperature. The tubes were placed on a magnetic rack for 4 minutes to collect the beads. Carefully, the supernatants were removed and discarded without disturbing the beads. 400 µl of freshly prepared 80% v/v ethanol was added leaving the tubes on the magnetic rack. After 30 seconds of incubation, the supernatants were discarded. The ethanol wash was repeated once more. The bead pellets were air-dried for 5-30 minutes until no more droplets of ethanol were visible. The beads were resuspended in 50 µl of 10 mM Tris-HCl pH 8 by pipetting up and down at least 20 times. The tubes were incubated for 10 minutes at room temperature and placed on a magnetic rack for 4 minutes to capture the beads. Carefully, 48 µl of supernatant of each tube were transferred to a new tube and store at -20°C.

4.2.4 Yeast-two-hybrid assays

pGADT7 and *pGBKT7* vectors (Clontech) were digested with BamHI-HF and EcoRI-HF. For *pGADT7* vectors, the coding sequences of *PavDAM4* (0.66kb), *PavDAM5* (0.7kb), *PavDAM6* (0.71kb), and *PavFT* (0.38kb) were amplified from flower buds of 'Burlat' cDNA using a Phusion® High-Fidelity DNA Polymerase (M0530S, NEB) with primers 13156+13157, 13160+13161, 13164+13165 and 13168+13169, respectively (Appendix Table 11). For *pGBKT7* vectors, the coding sequences of *DAM4*, *DAM5*, *DAM6* and *FT* were amplified with primers 13154+13155, 13158+13159, 13162+13163 and 13166+13167, respectively (Appendix Table 11). The amplicons were assembled with the *pGADT7-BamHI-EcoRI* or *pGBKT7-BamHI-EcoRI* fragments using Ligation-Independent Cloning (LIC). Positive clones were selected with kanamycin and carbenicillin for *pGBKT7* and *pGADT7*, respectively. *pGADT7* vectors were sequenced with primers 6161 and 13222 (Appendix Table 11), and *pGBKT7* vectors with primers 6483 and 13221 (Appendix Table 11).

Saccharomyces cerevisiae AH109 cells (Clontech) were transformed using the PEG/LiAc method as outlined by Clontech (2009b) and transformants were selected on synthetic drop-out medium lacking leucine and tryptophan (630317, Clontech) and screened on synthetic drop-out medium lacking leucine, tryptophan, histidine and adenine (630323, Clontech). The Yeast-Two-Hybrid system is explained in the Figure 4.7.

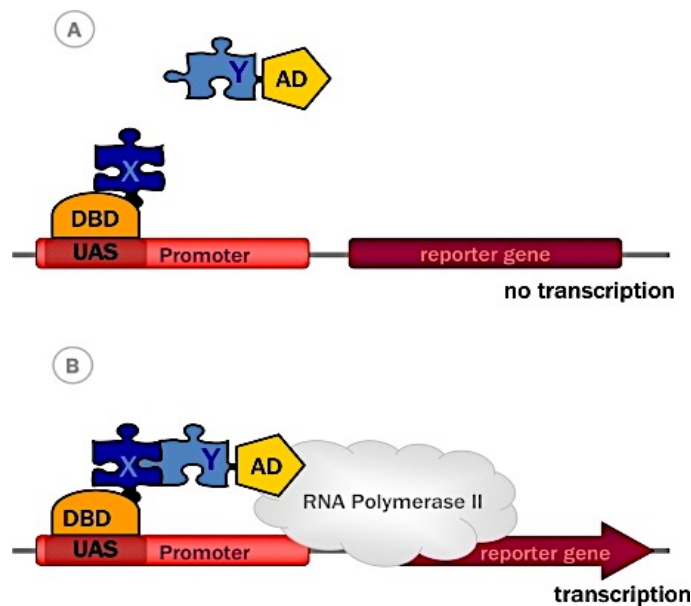


Figure 4.7 | Yeast Two Hybrid system

(A) The protein of interest X, is fused to the DNA binding domain (DBD), a construct called bait. The potential interacting protein Y is fused to the activation domain (AD) and is called prey. (B) The bait, i.e. the DBD-X fusion protein, binds the upstream activator sequence (UAS) of the promoter. The interaction of bait with prey, i.e. the AD-Y fusion protein, recruits the AD and thus reconstitutes a functional transcription factor, leading to further recruitment of RNA polymerase II and subsequent transcription of a reporter gene (Brückner *et al.* 2009).

4.2.5 Trans activation/repression assay

pGBKT7 vector (Clontech) containing the *GAL4* binding domain were digested with BamHI-HF and EcoRI-HF. *GAL4* activation domain and *GAL4* activation domain fused to *PavDAM6* from vectors previously created for a Yeast Two Hybrid assay, were amplified with primers 13394+13395 (DBD_{GAL4}::AD_{GAL4}) and 13394+13397 (DBD_{GAL4}::AD_{GAL4}::PavDAM6) (Appendix Table 12). The amplicons were assembled with the *pGADT7-BamHI-EcoRI* fragments using Ligation-Independent Cloning (LIC). Positive clones were selected with kanamycin. *pGBKT7* vectors were sequenced with primers 6161 and 13221.

Saccharomyces cerevisiae AH109 cells (Clontech) were transformed using the PEG/LiAc method as outlined by Clontech (2009b) and transformants were selected on synthetic drop-out medium lacking tryptophan (630308, Clontech). The Trans activation/repression system is explained in the Figure 4.8. The quantification of the beta-galactosidase were carried out following the procedure outlined by Clontech (2009) using a spectrophotometer.

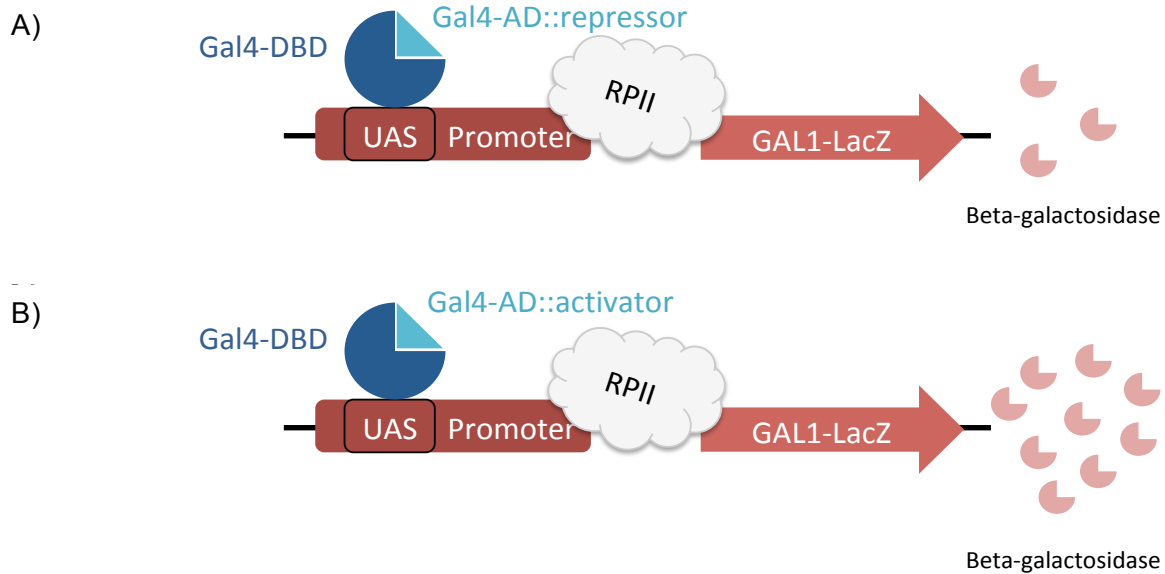


Figure 4.8 | Trans activation/repression system

Two different functional domains of Gal4 were used: an N-terminal DNA binding domain (DBD) and a C-terminal (transcriptional) activation domain (AD), with both individual domains maintaining their function independent of the presence of the other. One vector contains AD and DBD domains which reconstitute a functional Gal4 transcription factor. Gal4 then recruited RNA polymerase II, leading to transcription of a GAL1-lacZ fusion gene. This reporter gene encodes the enzyme beta-galactosidase which labels the yeast cell when using a colorimetric substrate. (a) A gene of interest is fused to AD domain, if the gene of interest plays a repressor role so the transcription of GAL1-lacZ fusion gene decreases as well as the enzyme beta-galactosidase. Conversely, (b) if the gene of interest plays an activator role so the transcription of GAL1-lacZ fusion gene increases as well as the enzyme beta-galactosidase.

4.2.6 Phytohormone quantification

I carried out the phytohormone extraction at the C.M.I (Roullier Group, St Malo in France) and Adrian Schwarzenberg, the metabolite platform manager at the C.M.I, carried out the phytohormone quantification using an UHPLC-MS/MS machine and performed sample analysis.

a) Phytohormone extraction

10 mg of frozen pulverised flower buds were weighed in a 2 ml tube. The extraction was carried out by adding 1 ml of cold 70% MeOH / 29% H₂O / 1.0% formic acid (stored at -20°C), containing isotopically labeled internal standards. Then, the tubes were stirred at room temperature for 30 min and centrifuged (5427 R, Eppendorf) at 16,000 rpm for 20 minutes at 4 °C. The supernatant of each tubes were transferred into new tubes and evaporated to dryness under nitrogen using a Turbovap LV system (Biotage, Sweden). The dried extracts were dissolved with 1 ml of a 2% formic acid solution. The resuspended

extracts were purified using a solid phase extraction (SPE) Evolute express ABN 1ml-30 mg (Biotage, UK). The eluate was evaporated to dryness and resuspended in 200 μ l of 0.1% of formic acid.

b) Quantification of phytohormones by UHPLC-MS/MS

Phytohormones such as salicylic acid (SA), jasmonic acid (JA), N[(-)-jasmonoyl]-(L)-isoleucine (JA-Ile), abscisic acid (ABA), abscisic acid glucosyl ester (ABA-GE), gibberellins (GA4, GA7, GA9), [2H4]-SA, [2H5]-JA, [2H6]-ABA, [2H2]-JA-ILE, [2H2]-GA4, [2H4]-1-aminocyclopropane carboxylic acid (D-ACC), were purchased from OlchemIn (Olomouc, Czech Republic). Dihydrophaseic acid (DPA), phaseic acid (PA), [2H3]-dihydrophaseic acid (D-DPA), and [2H3]-phaseic acid (D-PA) were purchased from NRC (Ottawa, Canada). 1-aminocyclopropane carboxylic acid (ACC) was purchased from Sigma-Aldrich (Saint-Quentin, France). Phytohormones were analysed by an UHPLC-MS/MS system. The separation and detection were achieved using a Nexera X2 UHPLC system (Shimadzu, Japan) coupled to a QTrap 6500+ mass spectrometer (Sciex, Canada) equipped with an electrospray (ESI) source. Phytohormones separation was carried out by injecting 2 μ l into a Kinetex Evo C18 core-shell column (100 x 2.1mm, 2.6 μ m, Phenomenex, USA) at a flow rate of 0.7 ml/min, and the column oven was maintained at 40°C. The mobile phases were composed of solvent A Milli-Q water (18 M Ω , Millipore, USA) containing 0.1% formic acid (LCMS grade, Fluka analytics, Germany), and solvent B acetonitrile LCMS grade (Fisher Optima, UK) containing 0.1% formic acid. The gradient elution started with 1% B, 0.0-5.0 min 60% B, 5.0-5.5 min 100% B, 5.5-7.0 min 100 % B, 7.0-7.5 min 1% B, and 7.5-9.5 min 1% B. The ionisation voltage was set to 5kV for positive mode and -4.5 kV for negative mode producing mainly [M+H]⁺ and [M-H]⁻ respectively. The analysis was performed in scheduled MRM mode in positive and negative mode simultaneously with a polarity switching of 5 ms. All quantitative data was processed using MultiQuant software V 3.0.2 (Sciex, Canada).

4.2.7 Sulphur metabolite quantification

I carried out the sulphur metabolite extraction at the C.M.I (Roullier Group, St Malo in France) and Adrian Schwarzenberg, the metabolite platform manager at the C.M.I, carried out the sulphur metabolite quantification using an UPLC-QToF machine and performed sample analysis.

a) Sulphur metabolite extraction

70 mg of frozen pulverised flower buds were weighed in a 2 ml tube and maintained in liquid nitrogen before processing to the next step. 500 µl of cold milliQ water/methanol 70:30 v/v (stored at -20°C) containing 0.4% of perchloric acid (v/v) solvent was added to the tubes and quickly mixed with a vortex (having a multiple sample head set) during 10 minutes. Then, samples were centrifuged (Centrifuge 5427 R, Eppendorf) at 12,700 rpm for 15 minutes at 4°C. 400 µl of each supernatant were carefully transferred into new 2 ml tubes without disturbing the pellet. A second extraction was performed on the remaining powder by adding 500 µl of 0.1% perchloric acid (v/v in milliQ water). Samples were mixed with a vortex during 5 minutes then, centrifuged at 12,700 rpm for 15 minutes at 4°C. Again, 400 µl of each supernatant were carefully transferred without disturbing the pellet to the tubes containing the previous supernatants. The tubes were inverted several times (or vortex) to homogenise the liquid. A centrifugation at 12,700 rpm for 10 minutes at 4°C was carried out in order to pellet suspended particles that may interfere with downstream processes. The supernatant of each tube were recovered into new 2 ml tubes without disturbing the pellet. Finally, supernatants were diluted 2fold with 0.1% formic acid (v/v in milliQ water) and transferred in 2 ml LC-MS vials.

b) Quantification of sulphur metabolites by UPLC-QToF

Ultra-high performance liquid chromatography analysis was performed using a Waters Acquity H-Class UPLC system (Waters Corp, Milford, USA). Sulfur-containing metabolites, GSH, GSSG, SAM, OAS, Met, separation was performed using a Waters UPLC HSS T3 column (2.1 x 100 mm, 1.8 µm). The mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile/methanol 50:50 v/v containing 0.1% formic acid (B) was applied with the optimised gradient elution as follows: 100% A at 0–1.5 min, 100–80% A at 1.5–2 min, 80–20% A at 2–2.5 min, 20% A at 2.5–4.5 min, 20–100% A at 4.5–5 min, 100% A at 5–7 min. The flow rate was kept at 0.4 mL/min, and column temperature was maintained at 25 °C.

High-resolution mass spectrometry detection of metabolites was performed by Waters Xevo G2-S quadrupole/time-of-flight mass spectrometer (QToF MS) (Waters Corp, Milford, USA) equipped with an electrospray ionization (ESI) source. Positive ESI source voltage was set to 0.5 kV and cone voltage was 15 V, whereas source temperature was maintained at 130 °C with a cone gas flow of 20 l/h. Desolvation temperature was at 500 °C with desolvation gas flow of 800 l/h. Leucine-Enkephalin (Waters, Manchester, UK) was used as lockmass reference, (ion at m/z 556.2771 in positive mode), which was introduced by a Lockspray at

10 μ /min for real-time data calibration. The MSE data were acquired in centroid mode using a scan range 50-800 Da, scan time 0.1 s, resolution was set at 20000 full width half maximum (FWHM), and a collision energy ramp 40-80 V. Molecular ions $[M+H]^+$ were detected in positive ionization. Chromatographic peaks were extracted from the full scan chromatograms using MassLynx V4.1 software (Waters Inc., USA), based on $[M+H]^+$ ions. Peak areas were integrated using TargetLynx software (Waters Inc., USA).

The transcriptome analyses in response to seasonal changes were investigated in sweet cherry floral buds using RNA-seq in order to determine the dormancy phases and transcriptional events during bud development. The transcriptome analyses for three cherry cultivars are presented in this following publication. The progress publication is well advanced and is in preparation for submission in BMC genomic journal.

5. Cherry bud developmental phases can be inferred from their transcriptional profiles

Noémie Vimont^{1,2,3}, David Schoepfer³, Mathieu Fouché¹, Varodom Charoensawan^{3,4,5,6}, Jean-Claude Yvin², Mustapha Arkoun², Elisabeth Dirlwanger¹, Sandra Cortijo^{3*}, Philip A. Wigge^{3*}, Bénédicte Wenden^{1*}

¹UMR 1332 BFP, INRA, Université de Bordeaux, F-33140 Villenave d'Ornon, France

²Agro Innovation International - Centre Mondial d'Innovation - Groupe Roullier, 35400 St Malo, France

³The Sainsbury Laboratory, University of Cambridge, CB2 1LR, United Kingdom

⁴Department of Biochemistry, Faculty of Science Mahidol University Bangkok Thailand

⁵Integrative Computational BioScience (ICBS) Center Mahidol University Nakhon Pathom Thailand

⁶Systems Biology of Diseases Research Unit, Faculty of Science Mahidol University Nakhon Pathom Thailand

5.1 Introduction

Plants are sessile organisms and must adapt their physiology to survive in a context of seasonal changes, such as temperature fluctuations over different seasons. This is particularly important for perennial plants because flowering occurs during the subsequent season exposing reproductive structures to high temperature variations, including cold during winter. One of the strategies to survive under low temperatures is a period of dormancy. This latent period has long been compared to an unresponsive physiological phase, in which metabolic processes within the buds are halted by cold temperature. However, several studies have shown that bud dormancy progression can be affected in a complex way by temperature and photoperiod (Heide and Prestrud, 2005; Svendsen *et al.*, 2007; Allona *et al.*, 2008; Heide, 2011; Cooke *et al.*, 2012).

Following the bud transition from a vegetative (dome shape) to a reproductive meristem (establishment of flower primordia) is marked by a shift in organogenetic activity in summer (Engin and Ünal, 2007), the growth cessation of active shoot occurs in late summer as the photoperiod and temperature decrease (Lang *et al.*, 1987; Balandier *et al.*, 1993; Campoy *et al.*, 2011), which marks the beginning of dormancy called (i) **paradormancy**, also named “summer dormancy” (Cline and Deppong, 1999). (ii) **Endodormancy** is initiated by lower temperature and shorter photoperiods in autumn (Lang *et al.*, 1987; Heide, 2008), associated with a cold acclimation process (Mølmann *et al.*, 2005; Ruttink *et al.*, 2007;

Tanino *et al.*, 2010). Endodormancy is deepened progressively (Rohde and Bhalerao, 2007) until reaching a stage at which bud development becomes unresponsive to growth-promoting temperatures. The chilling temperatures needed to overcome endodormancy are known as the chilling requirements (CR). (iii) Once the fulfilment of CR is completed, buds are able to resume their growth under warmer temperature conditions (i.e. **ecodormancy**). The warm temperatures needed for the achievement of bud growth to flowering in spring are known as the heat requirements (HR). Temperature, sometimes associated with photoperiod, controls each phenological phase during the bud life towards flowering. However perennials are affected in the current context of global warming and changes in phenology have been reported. Notably, advances in bud break and blooming dates in spring have been observed in the northern hemisphere, thus increasing the risk of late frost damages (Cannell and Smith, 1986, Badeck *et al.*, 2004; Menzel *et al.*, 2006; Fu *et al.*, 2015). On the contrary, in tropical and subtropical countries where the chilling temperatures that are required to overcome endodormancy are lacking, incomplete dormancy release has been observed leading to bud burst delay and low bud burst rate (Erez, 2000). These phenological changes directly impact the production of fruit crops, leading to large potential economic losses (Snyder and Melo-Abreu, 2005). Consequently, it becomes urgent to acquire a better understanding of bud responses to temperature stimuli in the context of climate change in order to tackle fruit losses and anticipate future production changes.

Recently, several studies have investigated the physiological and molecular mechanisms of bud-dormancy transitions in perennials using RNA sequencing technology, thereby giving an insight into potential pathways involved in dormancy. These results suggest that the transitions between the three bud dormancy phases (para-, endo- and eco-dormancy) are mediated by pathways related to phytohormone (Zhong *et al.*, 2013; Kumar *et al.*, 2016a; Khalil-Ur-Rehman *et al.* 2017; Chao *et al.*, 2017), carbohydrates (Min *et al.*, 2017), temperature (Ueno *et al.*, 2013; Pau *et al.*, 2014), photoperiod (Lesur *et al.*, 2015), oxygen species (Takemura *et al.*, 2015; Zhu *et al.*, 2015), water (Lesur *et al.*, 2015), cold acclimation and epigenetic regulation (Kumar *et al.*, 2016b).

These analyses are based on a small number of time points, mainly two or three, taken during the endodormancy, ecodormancy or dormancy release stages. However, it is likely that three sampling dates are insufficient to capture all the information about transcriptional changes occurring throughout the dormancy cycle as it corresponds to a chain of biological events rather than an on/off mechanism. Moreover, the likelihood to sample during dormancy release is low when harvesting just a few time points. It also increases the problem of comparison of biological processes between species as the results are time-dependant and might lead to controversial results. For example, opposite regulation of gene expression has been reported for gibberellin biosynthesis and catabolism during dormancy

(Karlberg *et al.*, 2010; Bai *et al.*, 2013; Yue *et al.*, 2017). In addition to the small number of time points, the RNA-seq studies are principally focused on one cultivar, which cannot fully characterise the pathways involved in dormancy since the timing for the response to environmental cues is cultivar-dependant (Campoy *et al.*, 2011; Wenden *et al.*, 2017). Notable exceptions are the transcriptome of two Japanese pear cultivars harvested at two dates (Takemura *et al.*, 2015) and of ten peach cultivars harvested at one date (Leida *et al.*, 2012b) during the dormancy period. These studies identified similar pathways as mentioned above, however having limited sampling dates makes it difficult to separate the transcriptional changes occurring during the dormancy cycle from short-term changes brought about by particular environmental conditions during harvesting, which may include cold spells.

Many unresolved questions remain: Are there big changes in the transcriptome during dormancy progression? What are the fine-resolution dynamics of gene expression related to dormancy? When is a pathway up- or down-regulated? Can we explain differences in the time-interval for dormancy release between cultivars based on transcriptional profiles? Can we identify a small set of marker genes to define the developmental stage of buds?

We conducted transcriptome analysis using next-generation sequencing of sweet cherry flower buds, which is a perennial species highly sensitive to temperature (Heide *et al.*, 2008). A time-course spanning the entire bud development from flower organogenesis in July to flowering in the spring was conducted to observe gene regulation in precocious bud development, which might impact paradormancy and endodormancy onset. Different cultivars displaying contrasting dormancy release dates and flowering times were used for this study in order to pinpoint precise pathways involved in the regulation of dormancy.

We find that the developmental stages of flower buds can precisely be defined by the transcriptome in all three cultivars, even if they display different dormancy release dates, suggesting that the transcriptome is largely related to the developmental stage of the buds. We also identify pathways that are specifically activated at different developmental stages, suggesting that such pathways might be controlling them. Additionally, this paper provides a list of marker genes to characterise dormancy phases that can be used as a decision-making tool to estimate the dormancy status.

5.2 Material and Methods

Plant material

Samples were collected from three sweet cherry cultivars (*Prunus avium* L.) displaying contrasted flowering date: 'Cristobalina', 'Garnet' and 'Regina', being very early, early and late flowering cultivars, respectively. All trees were grown in the same orchard located at the

Fruit Experimental Unit of INRA in Bourran, South West of France (44° 19' 56" N, 0° 24' 47" E) and under the same agricultural practices to remove environmental bias such as drought, rainfall and variation of temperature impacting the comparison of cultivars. Trees were grown on deep loamy soils, in mild winter regions with an average annual rainfall of 825 mm. For sampling procedure, ten-eleven dates spanning the entire period from flower bud organogenesis (July 2015) to bud break (March 2016) were chosen (Figure 5.1 and Suppl. Figure 5.1). For each date, flower buds were sampled on-site from three trees (two for 'Regina'), corresponding to biological replicates to decrease the plant specific effects. Upon harvesting, buds were flash frozen in liquid nitrogen and stored at -80°C prior to performing RNA-seq.

Measurements of bud break and estimation of the dormancy release date

Each fortnight between 16st November 2015 and 4th April 2016, three branches bearing floral buds were randomly chosen from 'Cristobalina', 'Garnet' and 'Regina' trees. Branches were incubated in water pots placed in a growth chamber (25°C, 16h light/ 8h dark, 60-70% humidity). The water was replaced every 3-4 days. After ten days under forcing conditions, the total number of flower buds that reached the BBCH stage 53 (Meier *et al.*, 1994; Fadón *et al.*, 2015) was recorded. The date of dormancy release is estimated as the date when the percentage of buds at BBCH stage 53 is above 50% after ten days under forcing conditions.

Climatic data and chill accumulation calculation

Daily and hourly mean temperatures were collected from the local weather station (INRA CLIMATIK, <https://intranet.inra.fr/climatik>, station 33533002). Chill and heat accumulation were calculated using the hourly temperatures. Chill accumulation, here chill portions (CP), was calculated from October 1st using the Dynamic chill model (Fishman *et al.*, 1987), which has previously been found to be the best fitting chill model among several tested models (Albuquerque *et al.*, 2008; Luedeling *et al.*, 2009). For heat accumulation, the growing degree hours (GDH) model was used (Anderson *et al.*, 1986).

RNA extraction and library preparation

Total RNA was extracted from 50-60 mg of frozen and pulverised flower buds using RNeasy Plant Mini kit (Qiagen) with minor modification: 1.5% PVP-40 was added in the RLT buffer. RNA quality was evaluated using Tapestation 4200 (Agilent Genomics). Only samples with RINe (RNA integrity number equivalent) superior or equivalent to 8.5 were used for RNA-seq. Library preparation was performed with 1 µg of total RNA using the TruSeq Stranded mRNA Library Prep Kit High Throughput (96 samples, 96 indexes, Illumina cat. no. RS-122-2103). DNA quality from libraries was evaluated using Tapestation 4200 (Agilent Genomics).

The libraries were sequenced on a NextSeq500, at the Sainsbury Laboratory Cambridge

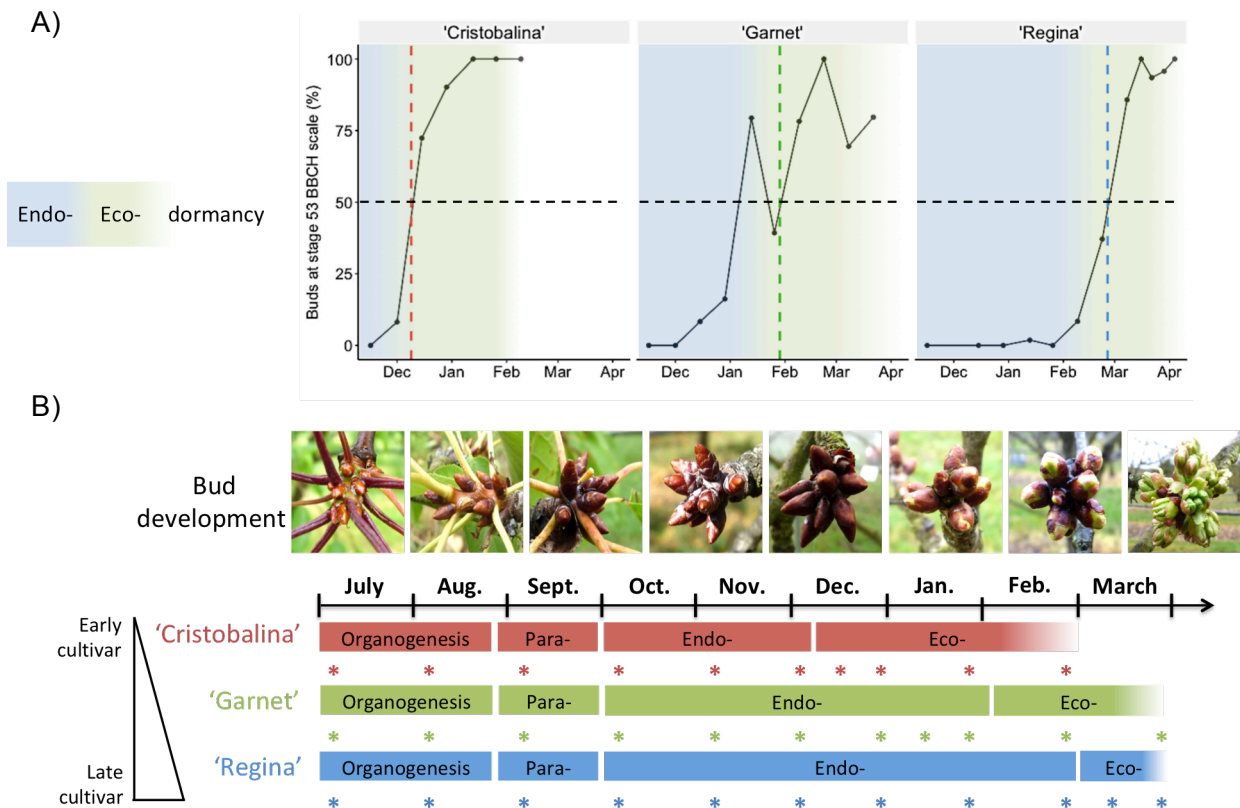


Figure 5.1 | Dormancy status under environmental conditions and RNA-seq sampling dates
 (A) Evaluation of bud break percentage under forcing conditions was carried out for three sweet cherry cultivars displaying different flowering dates in 'Cristobalina', 'Garnet' and 'Regina' for the early, medium and late cultivar, respectively. The coloured dotted line corresponds to the dormancy release dates, estimated at 50% of bud break. (B) Workflow of the harvest time points for RNA-seq represented by coloured stars. Red for 'Cristobalina', green for 'Garnet' and blue for 'Regina'

University (SLCU), using paired-end sequencing of 75 bp in length.

Data analysis

1. Mapping and differential expression analysis

The raw reads obtained from the sequencing were analysed using several publicly available software and in-house scripts. The quality of reads was assessed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and possible adaptor contaminations and low quality trailing sequences were removed using Trimmomatic (Bolger *et al.*, 2014). Trimmed reads were mapped to the *Prunus persica* reference genome v.2 (Verde *et al.*, 2017) using Tophat (Trapnell *et al.*, 2009) and possible optical duplicates were removed using v.2 (Verde *et al.*, 2017) Picard tools (<https://github.com/broadinstitute/picard>). Mapping metric of each samples are given in the **Suppl. Table 5.3**. For each gene, raw reads and TPM (Transcripts Per Million) were calculated (Wagner, 2003). Data were filtered by

removing lowly expressed genes based on expression level (TPM average < 3), genes not expressed in most samples and genes that have a little change in expression (coefficient of variation < 0.3). Differentially expressed genes (DEGs) were assessed using DESeq R Bioconductor package (Anders and Huber, 2010) on filtered data. Genes with an adjusted p-value (padj) < 0.05 were assigned as DEGs. Z-scores (i.e. (TPM-average)/STD) were calculated from TPM values assigned to DEGs.

2. PCA, gene clustering, GO enrichment analysis and marker genes analyses

Z-scores (i.e. (TPM-average)/STD) were calculated from TPM values assigned to DEGs. Pearson correlation analyses were carried out on DEGs, followed by a hierarchical clustering based on TPM values. Gene ontology enrichment was performed using the database resource PlantRegMap (<http://plantregmap.cbi.pku.edu.cn/>; Jin *et al.*, 2017). GO terms were filtered with a p-value < 0.01 and represented in barplot forms using $-\log_{10}$ (p-value). Predicted gene homology to Arabidopsis and gene functions were assigned to DEGs using available *Prunus persica*.v2 data files (ftp://ftp.bioinfo.wsu.edu/species/Prunus_persica/Prunus_persica-genome.v2.0.a1/homology/). The marker genes correspond to the most correlated genes to the mean data of each cluster with a correlation value > 0.9. Principal component analysis was performed on DEGs and marker genes using R.

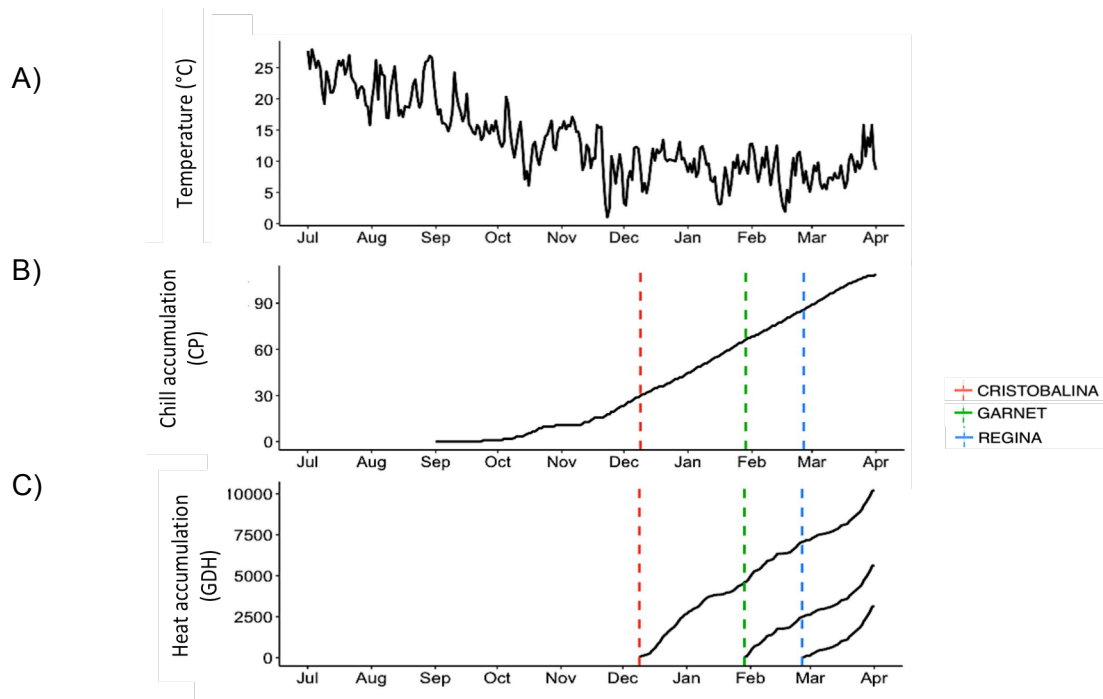


Figure 5.2 | Chilling and heat accumulation for the three sweet cherry cultivars

(A) Average daily temperature recorded on site during the sampling season 2015/2016. (B) Chill accumulated and (C) heat accumulated in chill portions (CP) and Growing Degree Hours (GDH) starting from the dormancy release of each cultivar during winter 2015-2016 for the three cultivars. The assumption is that heat accumulation starts when dormancy is released, after a given threshold for chill accumulation is reached. The coloured dotted line corresponds to the dormancy release dates evaluated using forcing experiments. Red for 'Cristobalina', green for 'Garnet' and blue for 'Regina'.

5.3 Results

5.3.1 Substantial differences in the dormancy release date and chill requirements between the three cherry cultivars

Endodormancy release dates were estimated for the three sweet cherry cultivars 'Cristobalina', 'Garnet' and 'Regina', which differ by two and a half months between the earliest and latest cultivars: 9th December 2015 for 'Cristobalina', 29th January 2016 for 'Garnet' and 26th February 2016 for 'Regina' (Figure 5.1 A). The difference in dormancy release date and flowering between cultivars is further associated with the determination of chilling requirements and heat requirements (Figure 5.2 B, C). For example, the early cultivar 'Cristobalina' is characterized by lower chill requirements and started accumulating warm temperatures earlier than the other cultivars (Figure 5.2 C). Therefore, by the end of the sampling sequence, the progression into ecodormancy was different for the three cultivars. According to bud break percentage (Figure 5.1 A), morphological observations (Figure 5.1 B), chilling and heat requirements (Figure 5.2) and descriptions from Lang *et al.* (1987), four stages were assigned to the samples: i) flower bud organogenesis in July and August, ii) paradormancy in September, iii) endodormancy in October to dormancy release (beginning of December for 'Cristobalina', beginning of February for 'Garnet' and end of February for 'Regina') and iv) ecodormancy starting from the estimated dormancy release date to flowering (Figure 5.1 B).

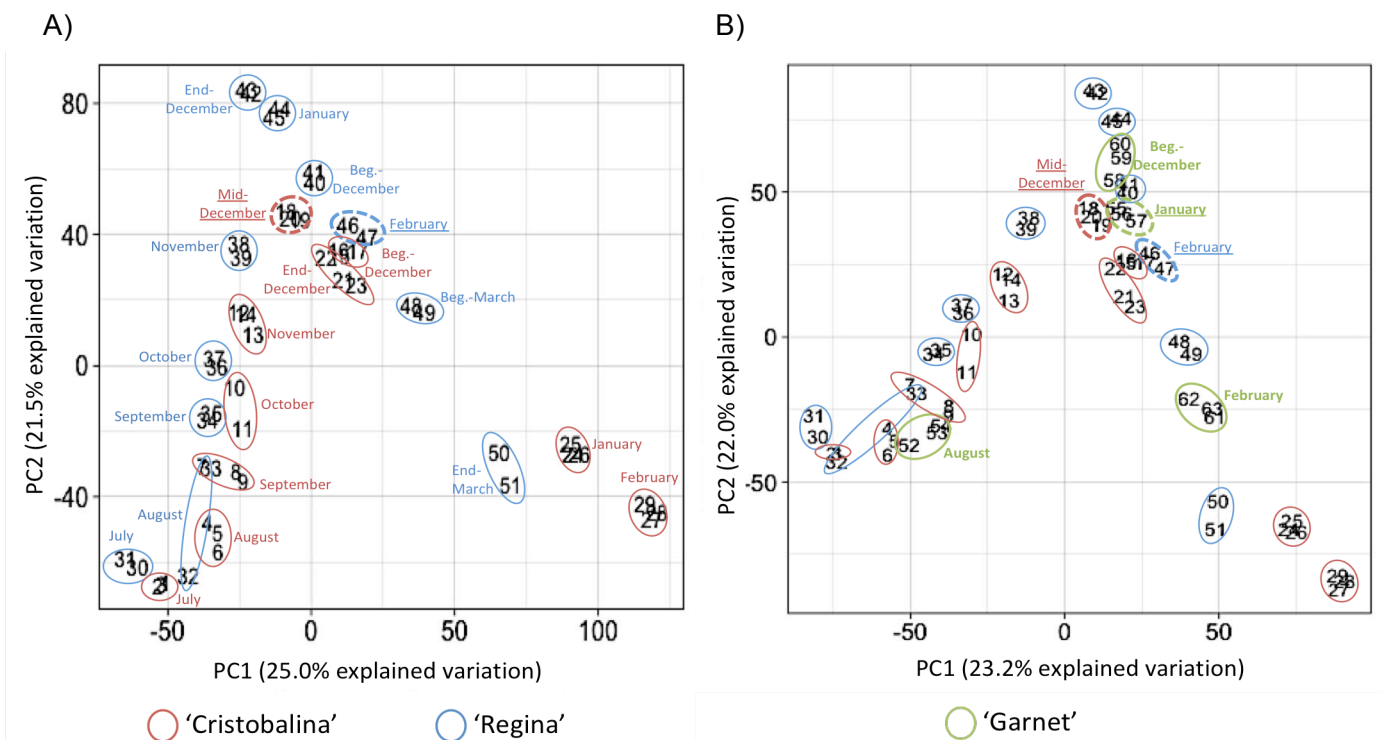


Figure 5.3 | Transcriptome accurately captures the dormancy state

(A) Principal component analysis of differentially expressed genes for 'Cristobalina', 'Regina' (B) and with a few samples of 'Garnet' throughout the sweet cherry flower development. Colour code: red for 'Cristobalina' (samples 1 to 29); blue for 'Regina' (samples 30 to 51) and green for 'Garnet' (samples 52 to 63). Dotted circles represent the dormancy release date of each cultivar.

5.3.2 Developmental stages of flower buds are characterized by the differentially expressed genes

We conducted a transcriptomic analysis on flower buds, performed by RNA-seq, from flower organogenesis in July to flowering in the spring spanning the entire bud development for the three cultivars. Of the 26,873 genes present in our transcript set, 11,355 genes were differentially expressed during the time-course in ‘Cristobalina’, 9,562 genes in ‘Garnet’ and 9,289 genes in ‘Regina’ (Suppl. Table 5.3).

In order to have a broad view of the transcriptomic data, we performed principal component analysis (PCA) using expression levels (TPM) of differentially expressed genes (DEGs) from the early cultivar ‘Cristobalina’ and the late cultivar ‘Regina’ across the different dates (Figure 5.3 A). First of all, the PCA revealed that replicates are clustered together, as was also observed during clustering analysis (Suppl. Figure 5.2). These results indicate that replicates are very similar even though buds come from different trees and also that the expression levels are not tree-dependent. An inverted V shape pattern arose from the different time points: starting from July on the bottom left, to December at the top and then February to the bottom right (Figure 5.3 A). The first dimension of the analysis (PC1) explained 25% of the variance and clearly defined the time, as the samples are ordered over months (Figure 5.3 A). The second dimension of the analysis (PC2) explains 21.5% of the variance and distinctly represents the bud dormancy status where samples at the bottom are non-dormant buds; samples in the middle are in transition between endodormancy and ecodormancy; and samples at the top are dormant (Figure 5.3 A). Samples from July to October for ‘Cristobalina’ and ‘Regina’ were clustered together suggesting that the transcriptional state of the buds is similar between the two cultivars at these dates. However in November, a separation between cultivars starts to be visible and becomes clear in December. Four contrasted time points were added to the component analysis from ‘Garnet’ an intermediate cultivar: August, December, January for which the dormancy release date was assigned, and February (Figure 5.3 B). The PCA obtained from the three cultivars was very similar to the first PCA (Figure 5.3 B). The dormancy release time points assigned to the three cultivars appeared to be clustered together (Figure 5.3 B). We thus show that the principal component analysis based on expression levels of buds accurately determines the bud state.

5.3.3 Expression patterns are associated with developmental stages

Having shown using PCA analyses that transcriptome of buds can define the bud dormancy state, we performed hierarchical clustering of differentially expressed genes based on their expression profiles. We identified ten main clusters of gene expression patterns

corresponding to groups of genes up-regulated throughout sampling dates (Figure 5.4). For example, two clusters correspond to genes up-regulated during flower bud organogenesis in July (cluster 10 dark green) and during ecodormancy (cluster 9, purple) (Figure 5.4). Clusters 1 (orange) and 2 (light blue) contain genes that show a peak of expression around the date of dormancy release in all three cultivars. Similarly, groups of genes activated at consecutive dates of the time-course are observed for each cultivar (Figure 5.5 A, Suppl. Figure 5.5). As we see the clearest organisation of the genes for ‘Regina’ (Figure 5.5 A), we used clusters identified in this cultivar as a reference for the rest of the study. We observed that 38.1% of DEGs (**Clusters 1 to 4**) are highly expressed during bud organogenesis, in July and August, and their expression diminishes during paradormancy in October. 14.5% of genes (**Clusters 5 and 6**) are expressed during paradormancy and endodormancy. 24.7% of genes (**Clusters 7 and 8**) show an increase of expression around the date of dormancy release. Finally 22.7% of genes (**Clusters 9 and 10**) show a peak of expression during ecodormancy, after dormancy release. Profiles of cluster 1, 3, 4 and 7 genes are the same in all three cultivars, suggesting that expression level of these genes are influenced mainly by the environment rather than by the developmental stage of the buds. On the contrary, the peak in expression for genes in cluster 8 is almost 2 months earlier in ‘Cristobalina’ compared to other cultivars, and slightly earlier in ‘Garnet’ compared to ‘Regina’, indicating that their expression is associated with the developmental stage of the buds.

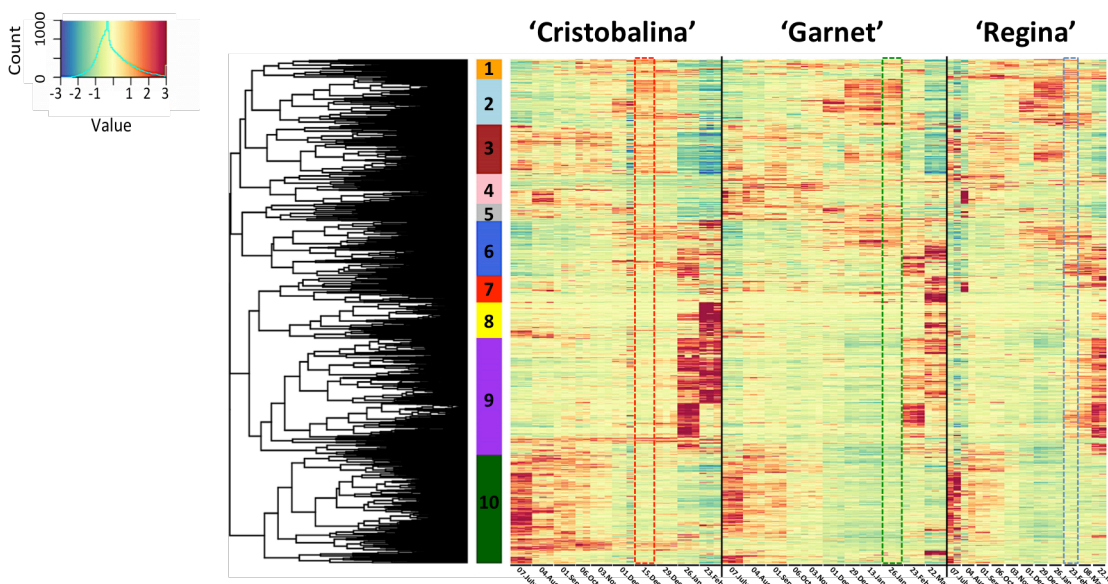


Figure 5.4 | Heatmap of differentially expressed genes in flower buds of three different sweet cherry cultivars during bud development

The heatmap was carried out using Z-score on ‘Cristobalina’ (early cultivar; n=3); ‘Garnet’ (intermediate cultivar; n=3) and ‘Regina’ (late cultivar; n=2). The horizontal rows from the heatmap represent genes, whereas the columns represent samples. Dotted lines represent dormancy release.

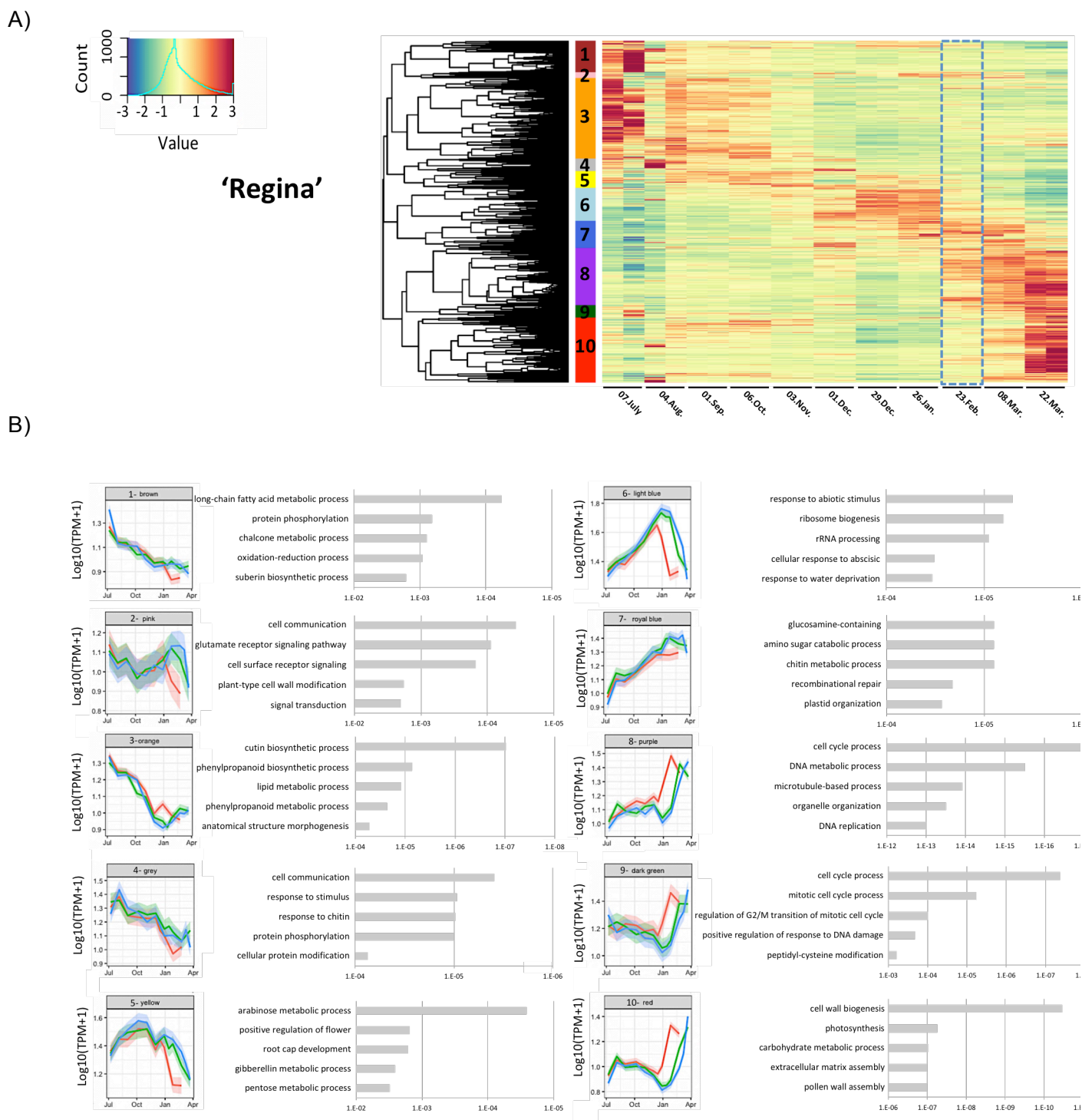


Figure 5.5 | Specific expression patterns during bud development

(A) The heatmap was carried out using Z-score on 'Regina' (late cultivar; n=2). The horizontal rows from the heatmap represent genes, whereas the columns represent samples. Dotted lines represent dormancy release. Cluster 1 counts 849 genes, cluster 2: 159 genes, cluster 3: 2,182 genes, cluster 4: 343 genes, cluster 5: 455 genes, cluster 6: 893 genes, cluster 7: 735 genes, cluster 8: 1,557 genes, cluster 9: 331 genes, cluster 10: 1,773 genes. (B) Gene ontology (GO) enrichment for different clusters of 'Regina'. Data are represented in $-\log_{10}$ (p-value).

5.3.4 Phase-dependent transcriptional activation and repression are associated with specific pathways

In order to identify pathways and functions that are specifically expressed at different stages of bud development, we performed a Gene Ontology (GO) enrichment analysis for each cluster (Figure 5.5).

In July-August (**Clusters 1, 2, 3 and 4**; Figure 5.5 A and B), main processes are activated such as cell communication and lipid metabolism while genes involved in sugar metabolism are activated during dormancy onset and their expression remain expressed onward (**Cluster 7**; Figure 5.5 A and B). During endodormancy, processes linked to the response to abiotic stimuli, including ABA, are strongly enhanced then inhibited after endodormancy release (**Cluster 6**; Figure 5.5 A and B). Some processes are activated from July to endodormancy release and then are down-regulated during ecodormancy, such as gibberellins metabolism (**Cluster 5**) or specifically activated during ecodormancy such as genes involved in growth recovery: cell cycle process and photosynthesis (**Clusters 8, 9 and 10**; Figure 5.5 A and B).

5.3.5 A set of marker genes can define the bud dormancy state

In order to characterise dormancy progression in a simple way, we proposed a set of ten marker genes, one for each cluster that was identified for 'Regina' (Figures 5.5 and 5.6). are highly correlated with the expression profiles of each cluster in the earliest and latest cultivars, 'Cristobalina' and 'Regina' respectively.

Among the ten genes, we identified four marker genes that are expressed during

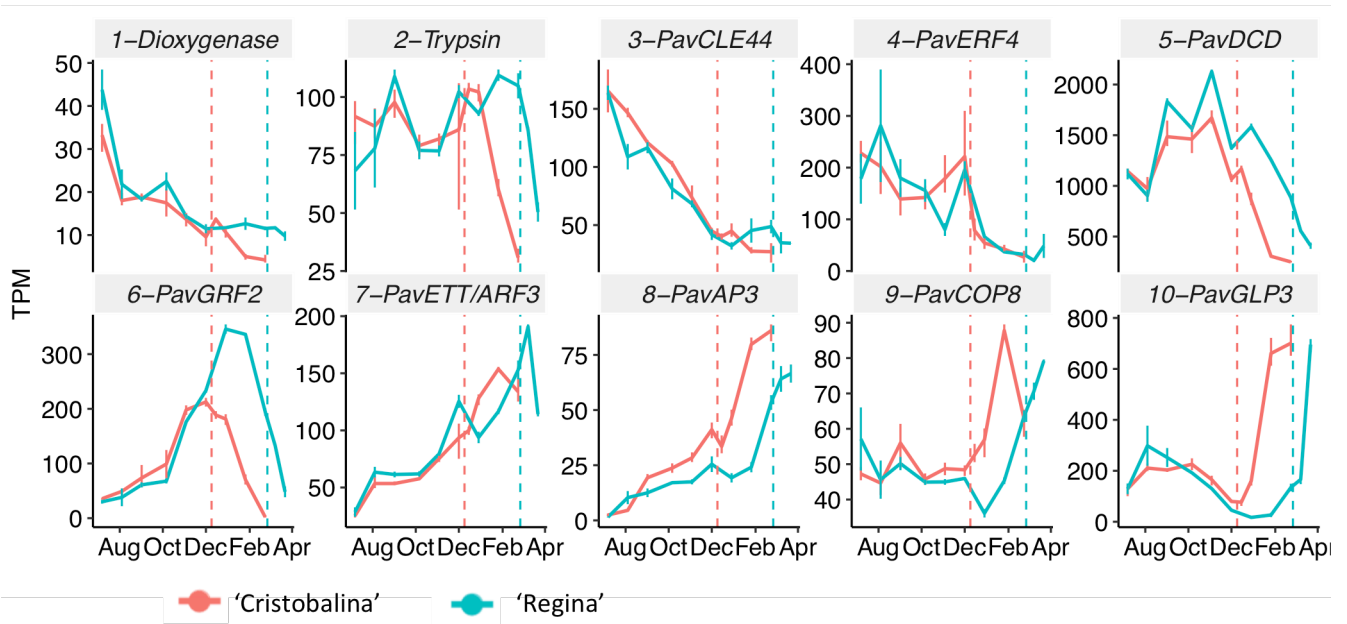


Figure 5.6 | Marker genes allow to distinguish each dormancy phase

Expression of specific genes involved in dormancy regulation are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the standard variation between the biological replicates (n=3 for 'Cristobalina', n=2 for 'Regina'). Dotted lines represent dormancy release.

precocious bud development and paradormancy: one gene encoding a dioxygenase (Cluster 1), *PavCLE44*, which is a homologue of *Arabidopsis thaliana* gene *CLAVATA3/ESR-RELATED44* (*CLE44*) (Cluster 3), one gene coding for a trypsin (Cluster 2) and *PavERF4*, a homologue of *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR4* (*ERF4*) (Cluster 4) (Figure 5.6).

Two marker genes of endodormancy onset to endodormancy release phases were identified from clusters 5 and 6: *PavDCD*, homologue of *DEVELOPMENTAL and CELL DEATH (DCD) domain protein*, and *PavGRF2*, homologue of *GROWTH REGULATOR FACTOR2* (*GRF2*) (Figure 5.6). The *PavGRF2* expression pattern was similar between cultivars from July to December and after this date the expression continues to rise in ‘Regina’ (late cultivar) while the expression decreased in ‘Cristobalina’ (early cultivar) after dormancy release (Figure 5.6).

To characterize the phase between endodormancy release and the end of endodormancy, four marker genes were selected, that are highly up-regulated from December until endodormancy: the *AUXIN RESPONSE TRANSCRIPTION FACTOR3* homologue *PavETT/ARF3*, the *APETALA3* homologue *PavAP3*, the *CONSTITUTIVE PHOTOMORPHOGENIC8* homologue *PavCOP8* and the *GERMIN LIKE PROTEIN3* homologue *PavGLP3* (associated with cluster 7, 8, 9 and 10, respectively (Figure 5.6).

A principal component analysis (PCA) was conducted on these ten genes for the three contrasted cultivars (Figure 5.7) and revealed a similar V shape pattern arising from the

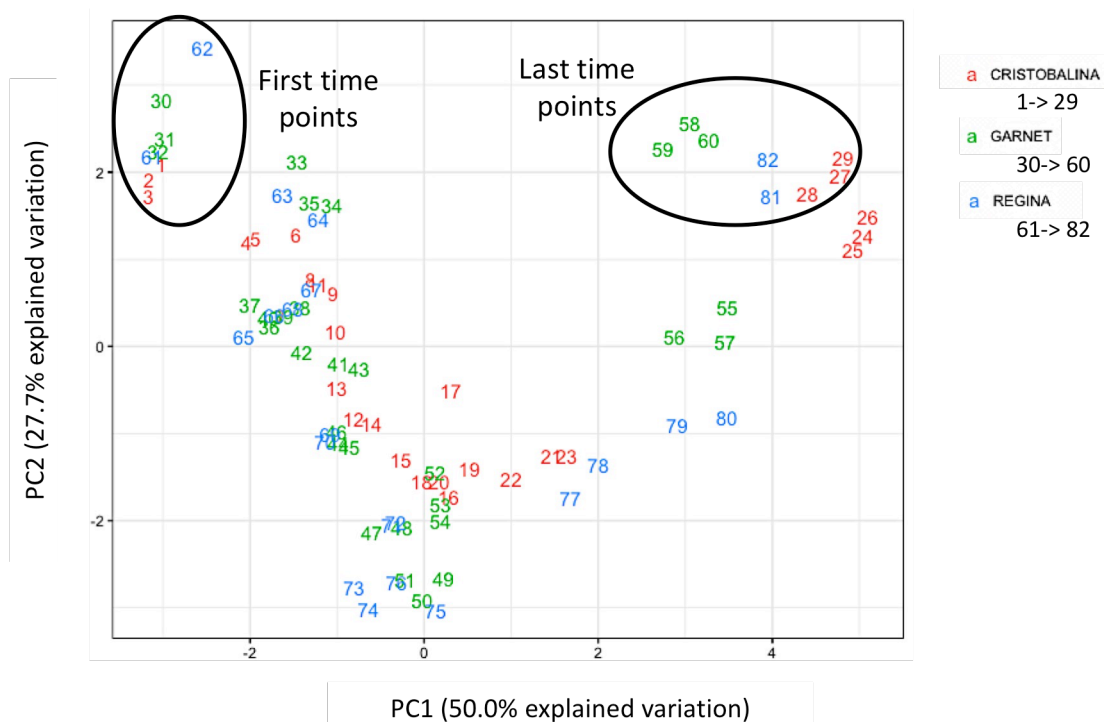


Figure 5.7 | Marker genes infer the sweet cherry developmental phase

Principal component analysis using ten marker genes. Colour code: red for ‘Cristobalina’ (samples 1 to 29), green for ‘Garnet’ (samples 30 to 60) and blue for ‘Regina’ (samples 61 to 82).

different time points than when the PCA was performed using around 30,000 genes (Figure 5.3). The first dimension of the analysis (PC1) explained 50.0% of the variance and the second dimension of the analysis (PC2) explained 27.7% of the variance and are also associated with the time and the dormancy status, respectively. Samples from 'Regina' in November were at the lowest position at the bottom of the PC2.

5.4 Discussion

5.4.1 Physiological states of the buds are associated with transcriptomic changes

The principal component analysis (PCA) performed on the transcriptome of three contrasted sweet cherry cultivars has led us to identify bud developmental phases based on time (PC1) and dormancy status (PC2) (Figure 5.3). In simple terms, samples in the upper part of the PCA are dormant whereas samples in the lower part are non-dormant. This is confirmed by the forcing experiments where samples before the dormancy release date are in endodormancy and correspond to the points in the upper part of the PCA, whereas samples exhibiting higher percentage of bud break are in the lower part of the PCA (Figure 5.3). The fact that physiological states of the buds can be separated by PCA is similar to results obtained in leafy spurge crown buds where para-, endo-, and ecodormant buds can be separated by performing a PCA on transcriptomes (Chao *et al.*, 2017). This demonstrates that the transcriptomic changes happening during dormancy are good proxy to separate samples according to the bud development stage.

Among such changes, we found an enrichment in genes involved in gibberellin metabolic processes in the cluster 5 with up-regulated genes from the precocious bud development to endodormancy onset then gradually decreased till April (Figure 5.5). This result is correlated with our findings that gibberellic acid (GA) levels increase during this same period (Chapter 8). GA biosynthesis-related genes in Chinese cherry were also found to be up-regulated before the accumulation of chill units (Zhu *et al.*, 2015) suggesting a key role for gibberellin in dormancy regulation. Abscisic acid (ABA), another hormone proposed to be involved in the regulation of dormancy in sweet cherry shown high level of ABA during deep dormancy and lower levels in ecodormancy associated with gene expression related to ABA biosynthesis (Chapter 8). These results are supported by similar recent findings in other perennials such as Japanese pear (Bai *et al.*, 2013), leafy spurge (Chao *et al.*, 2017), kiwi fruit (Wu *et al.*, 2017) and tea tree (Kumar *et al.*, 2016). We found an enrichment in cellular response to ABA in the cluster 6 for genes with an expression peak during deep endodormancy (Figure 5.5) confirming the significant role of ABA in dormancy maintenance in sweet cherry. In addition to ABA response, up-regulation of genes related to abiotic

stresses was reported for cluster 6, including water deprivation and abiotic stimulus. This is consistent with findings that the accumulation of transcripts linked to water deprivation is associated with ecodormancy (Ueno *et al.*, 2013; Lesur *et al.*, 2015). Indeed during this period, tissues are submitted to unfavourable conditions, potentially linked to climatic stresses such as dehydration and cold temperatures. The latter phase of ecodormancy is associated with growth resumption suggested by GO terms such as cell wall biogenesis, photosynthesis, mitotic cell cycle process and carbohydrate metabolic process (cluster 10; Figure 5.5 and cluster 10; Suppl. Figure 5.6). The crucial role of carbohydrate metabolism has been highlighted in sweet cherry dormancy with an increase in starch before bud break (Kaufmann and Blanke, 2017). The carbohydrate uptake capacity was shown to increase within the bud after dormancy release associated with an increase in the expression and activity of plasma membrane transporters leading buds to burst and growth (Beauvieux *et al.*, 2017).

5.4.2 New insights about dormancy phases between different cultivars

The principal component analysis for two contrasted sweet cherry cultivars 'Cristobalina' and 'Regina' has uncovered similarities and differences in transcript profiles. Samples in July and August corresponding to the precocious bud development are clustered together, suggesting that processes involved in the floral bud organogenesis are similar between cultivars. Samples from September and October are also clustered together, therefore we can hypothesise that the paradormancy phase occurs similarly between the two cultivars. Differences start to be visible between samples in November and become clear for samples in December. Samples in January for 'Regina', the late cultivar, are at the higher part of the PC2 defined as the dormancy status, while they are in the low part of the PC2 at the same date for 'Cristobalina', the early cultivar. We can therefore hypothesize that the late phenotype of 'Regina' is linked to a higher dormancy depth, initiated during early endodormancy.

Bud forcing experiments are used in multiple studies to estimate the endodormancy release date in various perennials (Yamane *et al.*, 2011; Leida *et al.*, 2012b; Wisniewski *et al.*, 2015; Takemura *et al.*, 2015; Ionescu *et al.*, 2017). This experiment has the advantage of being inexpensive and brings a quick overview of the dormancy state. However, the method is not standardised and considering dormancy release when 50% of buds are at 53 BBCH scale is arbitrary. We show that it is possible to correlate dormant and non-dormant samples with the PCA and the forcing experiment. These results indicate that the estimation of endodormancy release date by the forcing experiment when 50% of buds reached the stage 53 BBCH scale (Fadón *et al.*, 2015) is robust. In addition, samples from the three cultivars

estimated to be in endodormancy release are clustered together in the PCA (dotted circles) suggesting that the transcriptome state is similar between cultivars, potentially with the same regulation pathways activated during this crucial period, although shifted in time.

Interestingly, February samples from 'Regina' are clustered with samples for beginning of December from 'Cristobalina', corresponding to endodormancy release. For samples associated with ecodormancy, 'Cristobalina' January samples are close to March samples from 'Regina' (Figure 5.3 A) Therefore, although a delay is observed for dormancy release and flowering dates but transcriptomic profiles for samples corresponding to ecodormancy seem to be similar between cultivars.

This hypothesis allows us to reconsider the conclusion of studies based on two or more different cultivars in order to find pathways explaining the difference of dormancy release and flowering, as we cannot rule out that the differences identified in these studies are in fact caused by differences in the buds developmental stages. Leida *et al.*, (2012b) performed a microarray experiment on peach dormant flower buds of ten different cultivars harvested at a fixed date in late December in Spain. A gene coding for a putative late embryogenesis abundant protein (LEA) and *DORMANCY ASSOCIATED MADS-BOX (DAM)* genes appeared to be increased in late cultivars leading to late dormancy release phenotype. However, six out of ten cultivars displayed more than 50% of bud break in forcing conditions meaning that the majority of harvested buds were in endodormancy release or in ecodormancy. So according to our results, which suggest that the difference of dormancy release between cultivars is trigger during earlier dormancy phase suggesting that the generated list of genes from peach cultivars are the consequence of earlier changes in dormancy. Similarly to results reported in two different pear strains (high and low chilling strains), in which the expression probable *LEA* or *DEHYDRINS* increased in deep dormancy for samples harvested where the bud break percentage increased (Takemura *et al.*, 2015). We hypothesise that reported genes in these studies are involved in the regulation of dormancy but might not explaining the difference of dormancy release date phenotype.

5.4.3 Determination of bud developmental phases using a small gene subset: a decision-making tool for growers and researchers

In the context of global warming, the perennial response to warmer winter temperatures differs between cultivars and location. The effect of climate change on phenology in perennials has been reported mainly in regions of the northern hemisphere (Badeck *et al.*, 2004; Chmielewski and Rötzer, 2001; Menzel *et al.*, 2006; Fu *et al.*, 2015) constraining growers to adapt their agricultural practises or change tree species and cultivars (Darbyshire 2011, 2012; Luedeling 2009). For example, dormancy breaking reagents are used to compensate a lack of chilling and to synchronise flowering times. Winter chilling

accumulation is used by many growers to determine the optimal timing to apply dormancy breaking reagents and to predict fruit yield (Erez, 2000). Among the products available, hydrogen cyanamide is largely used to synchronise and accelerate bud break in several perennial crop species (Siller-Cepeda *et al.*, 1992; Sagredo *et al.*, 2005; Vegara *et al.* 2012; Zhou *et al.*, 2012, Wang *et al.* 2017; Ionescu *et al.*, 2017). One major factor limiting the use of hydrogen cyanamide is the date of application, since incorrect timing of application can lead to bud damages and this causes a decrease in production (Or, 1999). We suggest that transcriptomic profiles of marker genes for dormancy status can help set a precise date for the application of such reagents. The combination of these ten genes allows us to define each phase of dormancy (Figure 5.6):

- Precocious bud development and paradormancy phases are associated with high expression of *PavCLE44*, *PavERF4* and genes encoding dioxygenase and trypsin. Trypsin cleaves peptide chains and plant dioxygenases play an important role in the biosynthesis of plant signalling compounds such as ABA, GAs, and ethylene (Prescott and John, 1996). In *Arabidopsis*, *CLE44* functions as a suppressor of stem cell differentiation and is involved in axillary bud formation (Ito *et al.*, 2006; Hirakawa *et al.*, 2008). *AtERF4* acts as transcriptional repressor to modulate ethylene and ABA responses (Yang *et al.*, 2005) and operates in the progression of leaf senescence (Koyama *et al.*, 2013). Taken together, the early phase of dormancy seems to be highly regulated by phytohormones, including phytohormones biosynthesis (trypsin and dioxygenase activity) and responses (*PavERF4*).

- Endodormancy is associated with an upregulation in *PavGRF2* and *PavDCD*. The early phase endodormancy is correlated with high expression of *PavDCD*, which may play a role in cell death in cherry buds to produce scales that protect reproductive tissues during unfavourable growth conditions. Subsequently, *PavGRF2* is highly expressed during the late endodormancy phase and, since it is involved in leaf and cotyledon growth in *Arabidopsis thaliana* (Kim and Kende, 2004; Kim *et al.*, 2003), it may be associated with early flower formation within the bud (Fadón *et al.*, 2015).

- Ecodormancy is associated with enhanced expression of *PavETT/ARF3*, *PavAP3*, *PavCOP8* and *PavGLP3*. In *A. thaliana*, auxin response factors (ARFs) such as ARF3 are involved in auxin signalling (Tantikanjana and Nasrallah, 2012; Simonini *et al.*, 2017). *AP3* is a MADS-box gene required for the development of petals and stamens in the *Arabidopsis* flower (Riechmann *et al.*, 1996, Tilly *et al.*, 1998). An *et al.* (2011) demonstrated that ectopic expression of a poplar *APETALA3-like* gene in tobacco causes early flowering and fast growth. *PavAP3* is up-regulated during endodormancy release and ecodormancy during cherry flower organs development (Fadón *et al.*, 2015), suggesting a similar role for *PavAP3* in flower development in sweet cherry. In addition to increased expression of

PavAP3, *PavGLP3* was up-regulated during ecodormancy. In contrast to its name, the Arabidopsis *GERMIN-LIKE PROTEIN3 (GLP3)* is not involved in germination but in cell expansion and resistance to stress (Membré *et al.*, 1999). In *A. thaliana*, COP8 functions to suppress the photomorphogenic developmental program and to promote skotomorphogenesis (i.e. development of seedlings in the dark) (Kwok *et al.*, 1996, Wei *et al.*, 1994). *PavCOP8* expression peaks in 'Cristobalina' (early cultivar) during ecodormancy then decreases in February (Figure 5.6) while the expression in the late cultivar continues to rise (Figure 5.6). The increase in expression is associated with morphologically closed buds surrounded by dark scales and the decrease in expression is associated with open bud and with green sepals, therefore suggesting that skotomorphogenesis might be occurring during ecodormancy before bud break.

Overall, the expression levels for these ten genes could potentially be used to assess the dormancy state of cherry buds. Analysing the expression level of these genes by qRT-PCR would provide a fast and reliable tool for growers, to infer decisions such as timing for dormancy-breaking reagent application, similarly to services proposed by the company NSure (www.nsure.eu). In addition, this method to assess the dormancy status is less destructive than forcing experiment on cut branches since qRT-PCR is performed on a very small amount of biological material.

5.5 Conclusion and on-going analyses

High-throughput techniques on *A. thaliana* have led to a better understanding of pathways controlling various developmental phases. However, how these pathways are regulated under natural (non-laboratory) conditions, and how these molecular events can be transferred and compared with field plants, remain unclear. Recent studies highlighted the importance in finding molecular and physiological markers in each phase of dormancy transitions to model these dynamic processes under natural conditions (Kudoh 2016; Chuine and Régnière, 2017). Our analyses bring molecular outputs in the regulation of bud development *in natura* as our investigation represents the first reported study comparing several cultivars across a long time course spanning the entire 'life' of a flower bud. Our results have led to the discovery that the transcriptome can define precisely the bud dormancy status and that the difference in dormancy release date between cultivars is controlled by early processes found in November, when a divergence of transcriptome profiles is visible between the two contrasted cultivars. A more precise analysis will be carried out on November time points, to find a set of genes that might explain the difference in dormancy release and flowering dates between the three cultivars. In addition, one option

to further study transcriptome state as a proxy for dormancy progression is to conduct pseudotime analysis, which reveals temporal ordering of samples placing them along a trajectory corresponding to a biological process. Furthermore, our analyses may provide a great source of information to develop predictive models, which will be powerful tools to assist the breeding strategies.

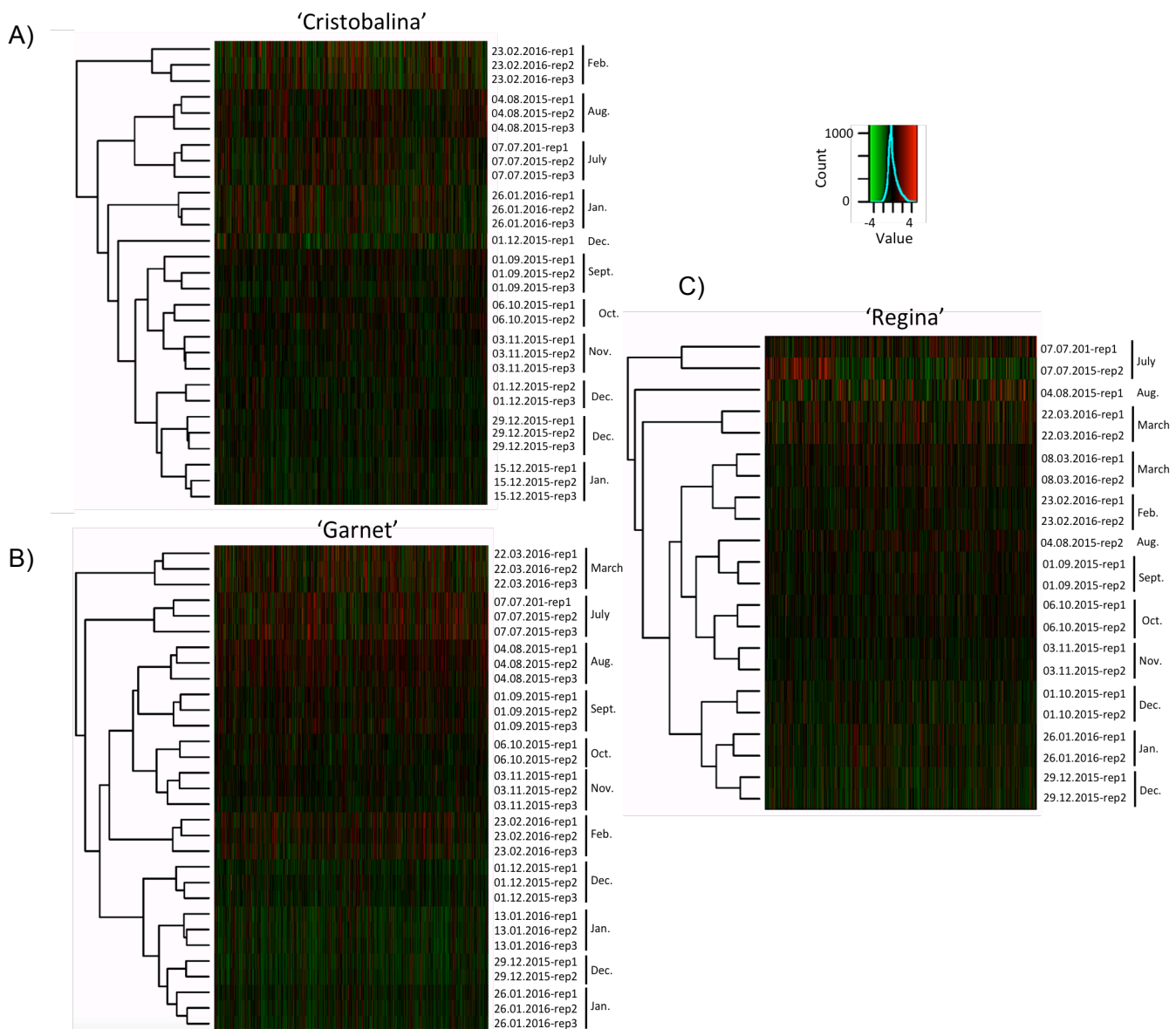
Our results strongly suggest that long time-series are essential to fully explore pathways or genes involved in dormancy release, especially for a precise determination of the dormancy status.

Finally, we will develop a web application in order to provide the research community with a straightforward rapid tool that will allow for the visualisation of candidate gene expression profiles for the three cultivars during dormancy.

Supplemental figures

Harvest Date	Varieties	Dormancy state
07 th July 2015	'Cristobalina', 'Garnet', 'Regina'	D, D, D
04 th August 2015	'Cristobalina', 'Garnet', 'Regina'	D, D, D
01 st September 2015	'Cristobalina', 'Garnet', 'Regina'	D, D, D
06 th October 2015	'Cristobalina', 'Garnet', 'Regina'	D, D, D
03 rd November 2015	'Cristobalina', 'Garnet', 'Regina'	D, D, D
01 st December 2015	'Cristobalina', 'Garnet', 'Regina'	D, D, D
15 th December 2015	'Cristobalina'	ND
29 th December 2015	'Cristobalina', 'Garnet', 'Regina'	ND, D, D
13 th January 2016	'Garnet'	D
26 th January 2016	'Cristobalina', 'Garnet', 'Regina'	ND, ND, D
23 th February 2016	'Cristobalina', 'Garnet', 'Regina'	ND, ND, ND
08 th March 2016	'Regina'	ND
22 th March 2016	'Garnet', 'Regina'	ND, ND

Suppl. Figure 5.1 | RNA-seq harvest time points

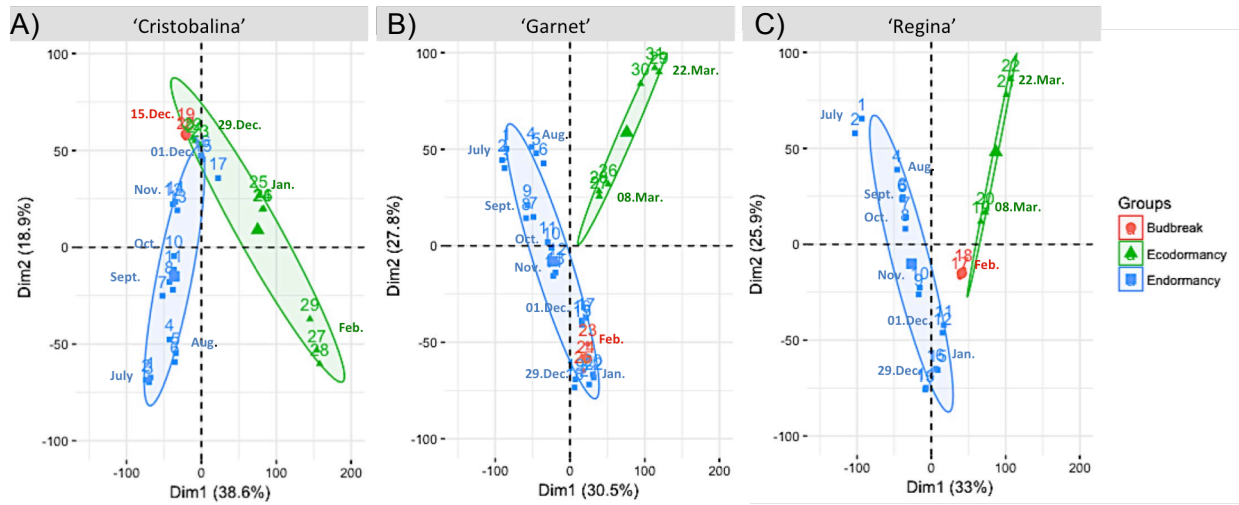


Suppl. Figure 5.2 | Heatmap of genes for A) 'Cristobalina', B) 'Garnet' and C) 'Regina'

Hierarchical clustering of transcriptomes of A) 'Cristobalina', B) 'Garnet' and C) 'Regina'. Each row represents a date and each column a gene. Z-scores of TPM values are represented in red for up-regulated genes or green for down-regulated genes, for a total of 8,679 genes. Clustering was carried out using complete agglomeration and Euclidean distance.

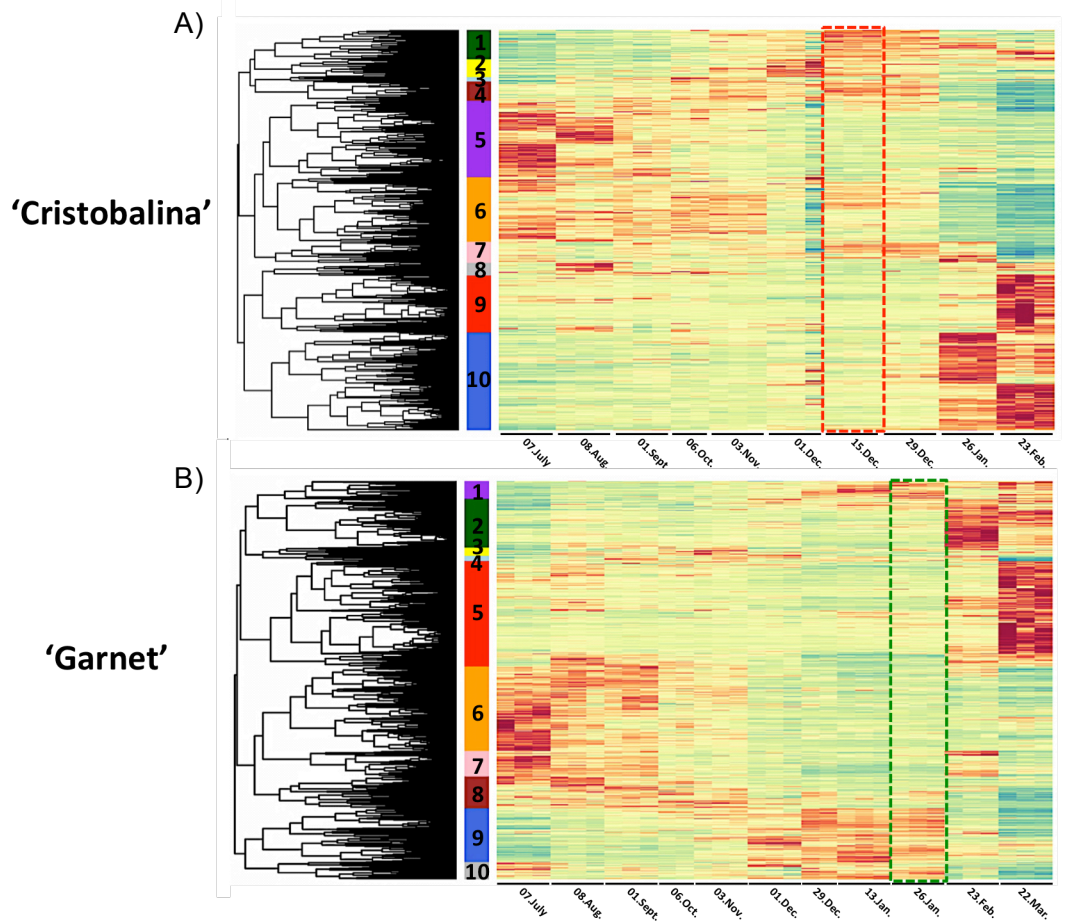
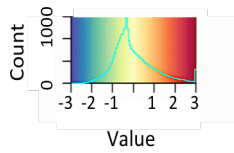
		'Cristobalina'			'Garnet'			'Regina'	
Mapped data	Date	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2
	07-July	11,492,765	17,857,105	11,932,765	13,269,736	11,129,047	9,401,179	14,515,028	3,8230,41
	04-Aug.	17,610,752	10,454,200	14,321,514	21,255,972	22,150,164	/	16,700,741	29,198,664
	01-Sept.	21,202,064	14,978,177	19,100,766	18,233,614	15,504,850	16,308,975	17,318,553	15,219,865
	06-Oct.	18,036,703	17,367,272	16,700,183	/	15,905,335	14,458,701	16,621,252	18,272,921
	03-Nov.	15,183,832	16,150,820	11,585,030	13,392,413	15,946,916	19,087,045	13,677,722	13,625,016
	01-Dec.	16,966,061	15,378,517	12,991,527	16,108,071	13,970,637	14,431,312	16,608,511	16,668,124
	15-Dec.	17,476,684	19,363,982	20,982,594					
	29-Dec.	20,277,333	19,300,574	18,228,456	18,369,337	18,853,626	/	14,515,355	16,684,489
	13-Jan.				16,787,134	19,948,427	15,768,071		
	26-Jan.	18,687,993	18,797,808	19,485,210	16,153,213	16,948,414	16,529,215	15,927,466	16,495,458
	23-Feb.	18,294,641	16,432,496	18,822,893	16,303,770	17,289,010	17,632,264	18,529,564	16,656,884
	08-Mar.							19,238,272	18,663,233
	22-Mar.				15,744,561	16,863,752	15,975,192	18,038,047	17,767,246
	Number of genes	Raw data	26,873			26,873			26,873
Remove lowly expressed genes		20,918			20,615			20,580	
Remove zeros		20,679			20,536			20,510	
Remove genes that have little change in expression		14,811			13,224			15,242	
DEseq		11,355			9,562			9,289	

Suppl. Table 5.3 | RNA-seq metrics from the three sweet cherry cultivars studied
 RNA-seq metrics from 'Cristobalina' (early cultivar), 'Garnet' (intermediate cultivar) and 'Regina' (late cultivar).



Suppl. Figure 5.4 | Transcriptome accurately captures the dormancy state for the three different sweet cherry cultivars

Principal component analysis of differentially expressed genes for (A) 'Cristobalina', (B) 'Regina' and (C) 'Garnet' throughout the sweet cherry flower development. Colour code: red for samples in budbreak phase, blue for samples in endodormancy phase and green for samples in ecodormancy phase.



Suppl. Figure 5.5 | Heatmap of differentially expressed genes in flower buds of (A) 'Cristobalina' and (B) 'Garnet' sweet cherry cultivars during bud development.

The heatmap was carried out using Z-score on 'Cristobalina' (early cultivar; n=3) and 'Garnet' (intermediate cultivar; n=3). The horizontal rows from the heatmap represent genes, whereas the columns represent samples. Dotted lines represent dormancy release.

We demonstrated in the Chapter #5 that the transcriptional changes occurring throughout dormancy is a chain of definable biological events. In this new chapter, we observed the dynamic of specific genes (such as *DORMANCY ASSOCIATED MADS-BOX* and *FLOWERING LOCUS T* genes) involved in dormancy and flowering during bud development in different cultivars. Additionally, we investigated their potential interactions and their potential target genes by new approaches. The progress publication is in preliminary stage, more experiments are needed to validate our hypothesis.

6. New insights about *DORMANCY ASSOCIATED MADS-BOX (DAM)* genes in the regulation of dormancy in sweet cherry (*Prunus avium* L.) floral buds

Noémie Vimont^{1,2,3}, David Schoepfer³, Mathieu Fouché¹, Jean-Claude Yvin², Mustapha Arkoun², Elisabeth Dirlwanger¹, Sandra Cortijo^{3*}, Philip A. Wigge^{3*}, Bénédicte Wenden^{1*}

¹UMR 1332 BFP, INRA, Université de Bordeaux, F-33140 Villenave d'Ornon, France

²Agro Innovation International - Centre Mondial d'Innovation - Groupe Roullier, 35400 St Malo, France

³The Sainsbury Laboratory, University of Cambridge, CB2 1LR, United Kingdom

6.1 Introduction

Genomic and genetic researches on the peach mutant 'evg', which prevents shoot growth cessation and dormancy, have revealed that six *DORMANCY ASSOCIATED MADS-box (DAM)* genes are associated to this non-dormant genotype (Bielenberg 2004, 2008). These *DAM* genes were identified as homologues of *Arabidopsis thaliana* *SHORT VEGETATIVE PHASE/AGAMOUS-LIKE 24* MADS-box transcription factors (Bielenberg *et al.*, 2008; Yamane 2008 and 2011), characterised as regulators of the floral transition (Hartmann *et al.*, 2000; Michaels *et al.*, 2003). *DAM* genes are seasonally regulated and their expression seems to be correlated to the dormancy status. This seasonal regulation was mainly shown for *PpeDAM5* and *PpeDAM6* genes in peach, which are up-regulated during growth cessation and down-regulated by cold exposure during winter (Jiménez *et al.*, 2010), thus suggesting a role in establishing and maintaining endodormancy (Li *et al.*, 2009; Saito *et al.*, 2013). These expression patterns were confirmed in Chinese cherry (*Prunus pseudocerasus*; Zhu *et al.*, 2015) and in Japanese apricot (*Prunus mume*; Sasaki, 2011; Kitamura 2016, Zhao 2018a). Recently, Kitamura *et al.* (2016) demonstrated by yeast two-hybrid screening that PmDAM6 can interact in yeast with the homologue of *Arabidopsis*

SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*), which acts as an integrator of several floral inductive pathways (Onouchi *et al.*, 2000, Lee and Lee, 2010). In addition to the physical interaction, synchronised expression patterns were detected in *PmDAM6* and *PmSOC1* during dormancy release in flower buds, thus suggesting a role for these two genes in the regulation of dormancy transition to flowering in Japanese apricot flower buds. *FLOWERING LOCUS T* (*FT*) is another gene of major interest in the control of dormancy, with a potential role in growth cessation and endodormancy induction (Horvath, 2009; Hsu *et al.*, 2011). Indeed, expression analyses suggest that DAM proteins may negatively regulate the expression of *FT* in leafy spurge and sweet cherry (Hao *et al.*, 2015; Rothkegel *et al.*, 2017). In addition, Zhao *et al.*, (2018a) demonstrated that *PmDAM6* can also interact with *PmCBF1*, an orthologous of *Arabidopsis C-repeat-binding factor/dehydration responsive element-binding factor 1* (*CBF/DREB1*). *CBF/DREB1* genes play important roles in freezing tolerance and cold acclimation in *Arabidopsis* (Novillo *et al.*, 2004, 2007; Achard *et al.*, 2008), and in perennials such as peach (Wisniewski *et al.*, 2015) and in tea plant (*Camellia sinensis* (L.) O. Kuntze; Wang *et al.*, 2012). Despite these advances, the molecular function of the *DAM* genes in perennials is still poorly understood.

In this study, we sought to gain knowledge on the function of *PavDAMs* in sweet cherry by analysing interactions between proteins and with their targets. Our results suggest that *PavDAM* genes are associated with specific phases of dormancy in sweet cherry. *PavDAM* proteins can interact together and with themselves, *PavDAM6* seems to be a transcriptional activator and *PavDAM5* proteins may target abiotic stimulus-related genes.

6.2 Material and Methods

Protein sequences alignment and analysis of protein domains/motifs

Sequences for *PavDAM6*, 5 and 4 genes were amplified from cDNA of sweet cherry (*Prunus avium* L.) and sequenced (more details are provided in the “Materials and Methods” section, page 72). The determination of protein sequences were done using EMBOSS Transeq website (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) to convert coding sequencing to protein sequence. We performed a blast on *PavDAM6*, 5 and 4 protein sequences using Multiple Sequence Alignments on the BAR website (http://bar.utoronto.ca/ntools/cgi-bin/ntools_multiplealign_w_mview.cgi). The domain/motif analyses were carried out by the combination of ExPASy SIB Bioinformatics Resource Portal (https://myhits.isb-sib.ch/cgi-bin/motif_scan) and Pfam database (<https://pfam.xfam.org/>) (Finn *et al.*, 2016).

PavDAM gene expression profiles

RNA-seq was performed on floral buds during their development for three different sweet cherry cultivars displaying different dormancy release dates, 'Cristobalina', 'Garnet' and 'Regina' being early, intermediate and late cultivars, respectively. RNA sequencing procedure and analysis were previously described in the Chapter 'Cherry bud developmental phases can be inferred from their transcriptional profiles' (see page 86).

Yeast-two-hybrid assay

Details are provided in the "Materials and Methods" section (page 76).

In simple terms, yeast-two-hybrid assay allows to see the protein-protein interactions by testing their physical interactions in yeast. If the protein-protein interaction occurs, it will activate reporter genes enabling yeast to grow on selective media.

Trans activation/repression assay

Details are provided in the "Materials and Methods" section (page 72).

In simple terms, trans activation/repression assay allows to test the transcriptional activity (repressor or activator) of transcription factors. One vector contains the activation domain (AD) and the DNA binding domain (DBD) of GAL4, which reconstitute a functional GAL4 transcription factor. GAL4 activates a reporter gene encoding the enzyme beta-galactosidase, which labels the yeast cell when using a colorimetric substrate.

In vitro ChIP-seq

1. Procedure

Details are provided in the "Materials and Methods" section (page 73).

In simple terms, *in vitro* ChIP-seq assay allows to determine the target genes of one particular transcription factor. A tagged transcription factor (PavDAM5::FLAGx3) was mixed with sheared sweet cherry DNA and immunoprecipitated. The DNA bound to the tagged protein is precipitated and sequenced.

2. Bioinformatic analyses

Sequenced *in vitro* ChIP-seq data were analysed using several publicly available software and in-house scripts. The quality of reads was assessed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and possible adaptor contaminations and low quality trailing sequences were removed using Trimmomatic (Bolger *et al.*, 2014). The adaptor-trimmed reads were mapped to the sweet cherry (*Prunus avium* L.) genome v1.0 (Shirasawa *et al.*, 2017) using Bowtie2 (Langmead *et al.*, 2009). Possible optical duplicates were removed using Picard tools (<https://github.com/broadinstitute/picard>).

A peak calling was performed using MACS2 (Zhang *et al.*, 2008). MACS2 created a file with fold change of the IP with the homologue genes of *Arabidopsis thaliana*. We performed a

Gene Ontology (GO) enrichment using GOrrilla (<http://cbl-gorilla.cs.technion.ac.il/>) on these putative targets and GO terms are represented in barplot forms using $-\log_{10}(\text{p-value})$. Enrichment at *PavDAM5* loci is represented using the Integrative Genome Viewer (IGV; Robinson *et al.*, 2011) as a tool for visualising sequencing read profiles.

6.3 Results

6.3.1 DAM proteins sequences display domain/motif conservations

Dormancy associated MADs-box (DAM) genes have largely been reported to be involved in dormancy in many perennials (Horvath *et al.*, 2010; Yamane *et al.*, 2011; Leida *et al.*, 2012; De la Fuente *et al.*, 2015; Saito *et al.*, 2015; Mimida *et al.*, 2015). We predicted six *PavDAM* genes in the cherry genome. Multiple protein sequence alignments were performed to determine the homology between sweet cherry *PavDAM5*, 4 and 6 protein sequences and the conserved motifs. High homologies were observed between the three *PavDAMs* with

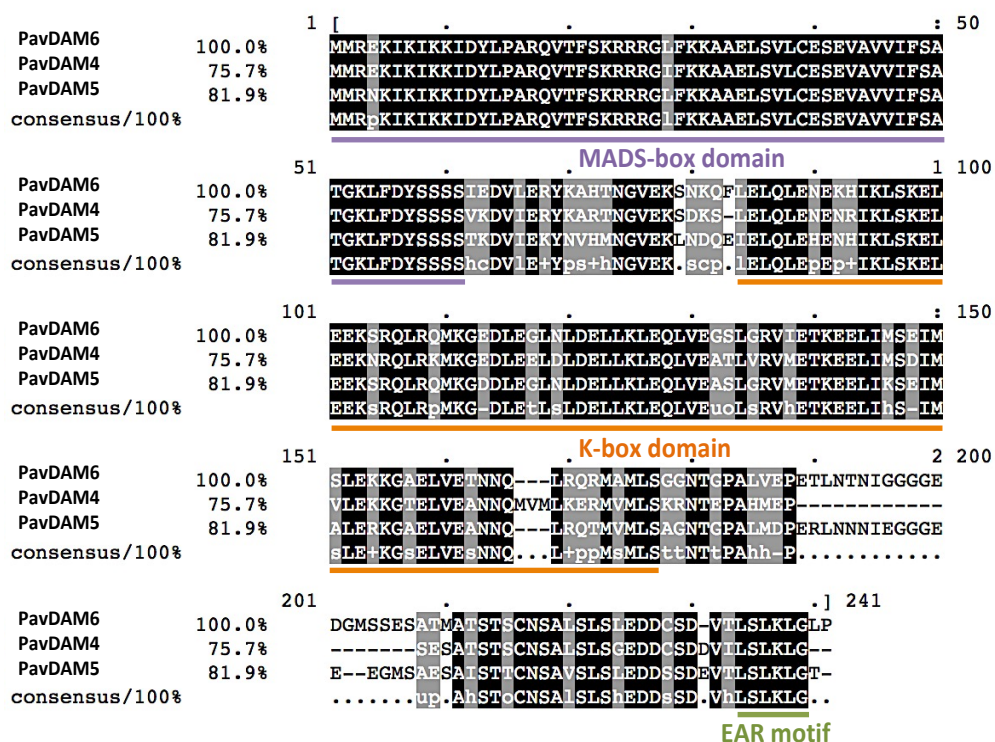


Figure 6.1 | Conserved domains along *PavDAM6*, 5 and 4 protein sequences in sweet cherry

Alignment of *PavDAM6*, 5 and 4 protein sequences. Three shared domains were found: MADS-box domain (purple), a K-box domain (orange) and a Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif (green).

75.7% of homology between *PavDAM6* and *PavDAM4*; and 81.9% of homology between *PavDAM6* and *PavDAM5* (Figure 6.1). The MADS-box domain was very well conserved and the Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR)

motif was exactly the same in the three proteins. The K-box domain displayed more differences between the three PavDAM protein sequences (Figure 6.1).

6.3.2 Waves of transcriptional activation and repression of *PavDAM* genes are observed during dormancy phases

We further analysed *PavDAM* expression profiles throughout dormancy progression. We find that six *DAM* genes (*PavDAM1-6*) show clear profiles of expression, indicating that they might be involved in dormancy (Figure 6.2). Consecutive peaks of *DAM* genes expression are visible: up-regulation of *PavDAM1*, *PavDAM3* and *PavDAM6* during dormancy onset, following by *PavDAM2* and *PavDAM5* during endodormancy, then *PavDAM4* before endodormancy release (Figure 6.2). The *PavDAM* expression trends are similar between cultivars, however the amplitude is lower in ‘Cristobalina’ for *PavDAM2*, *PavDAM5* and *PavDAM4*. Interestingly the peaks of expression for these genes in the two other cultivars occur at the same dates that are either shortly before (*PavDAM2* and *PavDAM5*) or after (*PavDAM4*) dormancy release in ‘Cristobalina’, while they are before dormancy release in ‘Garnet’ and ‘Regina’ (Figure 6.2). We therefore hypothesise that there is a positive correlation between the dormancy depth and the up-regulation of some *PavDAM* genes during endodormancy. Interestingly, the expression of *PavDAM* genes was not null in July during flower bud organogenesis suggesting a potential role of *DAM* genes in the precocious bud development.

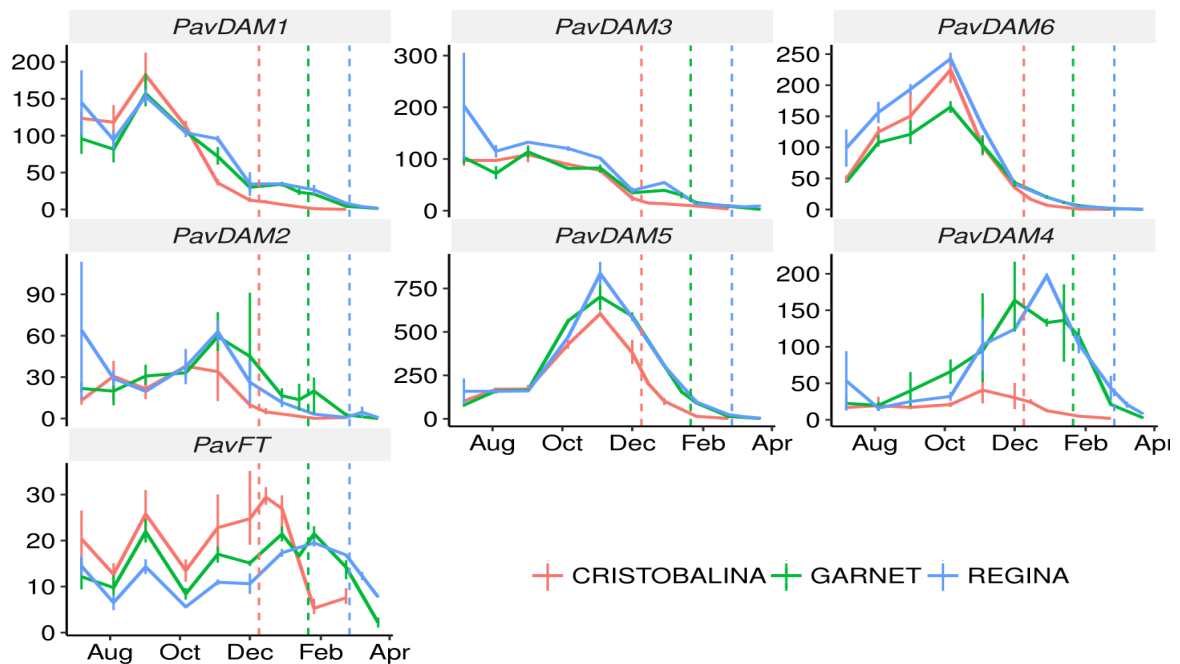


Figure 6.2 | Transcriptional dynamics of *PavDAM* and *FT* genes in the flower buds of three sweet cherry cultivars during bud development

Expressions are represented in TPM (Transcripts Per kilobase Million). Red: ‘Cristobalina’, green: ‘Garnet’ and blue: ‘Regina’. Error bars indicate the standard variation between the biological replicates (n=3 for ‘Cristobalina’, n=3 for ‘Garnet’ and n=2 for ‘Regina’). Dotted lines represent dormancy release dates (50% of budbreak).

6.3.3 Negative correlation between *PavFT* and *PavDAM* gene expressions during dormancy

Since it has been hypothesised that DAM proteins inhibit *FT* expression during dormancy, therefore preventing bud growth and development, we investigated *PavFT* expression in the three cultivars. Overall, expression for *PavFT* remains low over the whole bud development but a marked decrease is observable in October, concomitantly with up-regulation of *PavDAM5* and *PavDAM6* expression, which could suggest that these genes may be repressors of *PavFT* (Figure 6.2). *PavFT* expression then increases around the dormancy release date, when expression of *PavDAM* genes is repressed. Furthermore, *PavFT* is more expressed in 'Cristobalina' whereas expression for *PavDAM5* and *PavDAM4* is lower in this cultivar, thus corroborating the hypothesis that *PavDAMs* negatively regulate the expression of *PavFT*.

6.3.4 Physical interaction of *PavDAM* proteins

To investigate the potential interactions between *PavDAM* and *PavFT* proteins, we performed a yeast-two-hybrid assay for *PavDAM5*, *PavDAM4*, *PavDAM6* and *PavFT*. Sequences for each protein was fused to either the DNA-binding domain ('bait') or activation domain ('prey') of the yeast GAL4 transcriptional activator. All strains were efficiently grown on a non-selective media (-LW) as a positive control (Figure 6.3 A). On a selective media (-LWHA), no interactions were found between empty vectors, which confirm that no false positive might be present along the experiment (Figure 6.3 A). A weak interaction was observed between *PavDAM6* and *PavFT*, but not between other *PavDAMs* and *PavFT* (Figure 6.3 A). When used as the bait, *PavDAM5* interacted strongly with itself and with *PavDAM6*. Similarly, a strong interaction was observed when *PavDAM6* was used as the bait (Figure 6.3 A). In addition, we found that, as bait, *PavDAM6* can activate the reporter genes even in the absence of prey, unlike *PavDAM5* (Figure 6.3 B), although this interaction is weaker than when *PavDAM5* or *PavDAM6* are used as the prey. This suggests that *PavDAM6* has inherent activation potential.

6.3.5 *PavDAM6* acts as a transcriptional activator

Trans activation/repression assays were carried out to determine whether *PavDAM6* might be a transcriptional activator. A functional GAL4 transcription factor ($DBD_{GAL4}::AD_{GAL4}$) was used as a control and displayed a β -galactosidase activity (Figure 6.4). An effector plasmid was constructed by fusing *PavDAM6* with the DNA binding domain of the yeast GAL4 transcriptional activator ($DBD_{GAL4}::AD_{GAL4}::PavDAM6$). When the construction

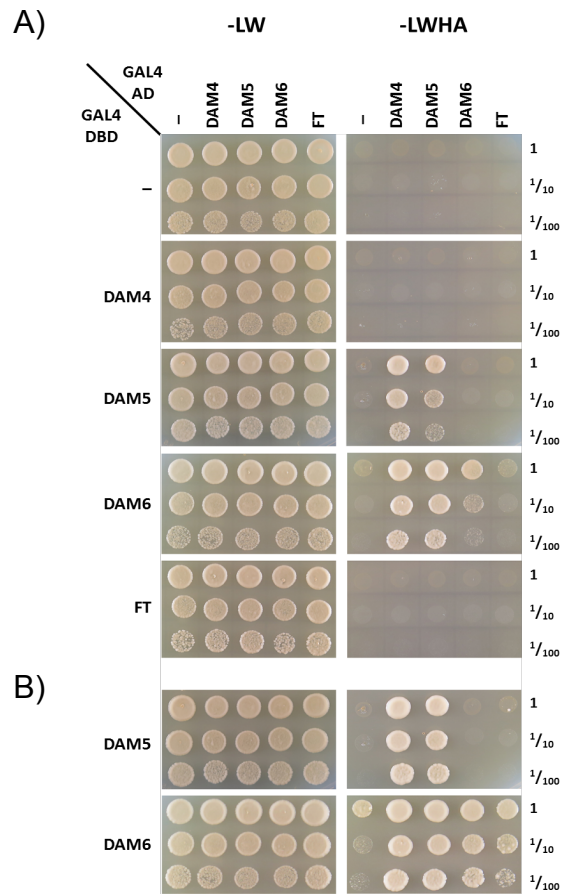


Figure 6.3 | Protein-protein interactions between PavDAMs and FT

Each protein was fused to either the DNA-binding domain (DBD, 'bait') or activation domain (AD, 'prey') of the yeast GAL4 transcriptional activator. Yeast strains were grown on a non-selective media (-LW) as positive controls, and then transferred to a selective media (-LWHA) to see the specific interaction between proteins. '-' sign corresponds to empty vectors, which are negative controls. Pictures were taken (A) after three days of growth at 30°C and (B) after five days at 30°C.

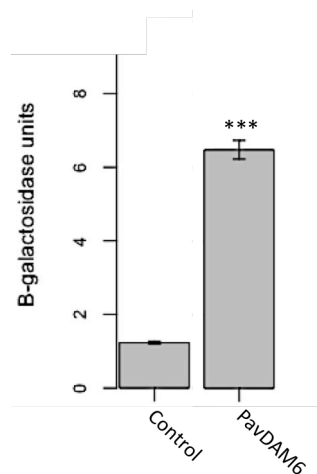


Figure 6.4 | PavDAM6 is an activator transcription factor

A trans repression/activation assay was carried out on $DBD_{GAL4}::AD_{GAL4}$ as a control compared to $DBD_{GAL4}::AD_{GAL4}::PavDAM6$. Error bars indicate the data range between the five biological replicates (n=5). Asterisks indicate that PavDAM6 differ significantly from control (Student test, $p \leq 0.001$).

DBD_{GAL4}::AD_{GAL4}::PavDAM6 was expressed in yeast strain, the β-galactosidase activity increased 5-fold relative to the control GAL4-AD (Figure 6.4). These results strongly suggest that PavDAM6 is a transcriptional activator.

6.3.6 Preliminary results of PavDAM5 *in vitro* ChIP-seq suggest a targeting of abiotic stimulus response genes

In vitro ChIP experiment was performed on PavDAM5 as a first trial to determine the potential target genes of DAM proteins. As we did not have an INPUT in this trial experiment, we could not perform an accurate peak calling to identify potential PavDAM5 targets. We thus selected genes with a fold enrichment superior to 5 as being putative targets (Figure 6.5), which we could use to confirm this first trial. Interestingly, one of these putative targets is *PavDAM5*, where we can identify a clear PavDAM5 signal in the promoter region and at the last exon (Figure 6.6). We then performed a Gene Ontology (GO) analysis on these putative targets, and identified an enrichment for RNA process and response to abiotic stimulus (Figure 6.5). Genes related to abiotic stimulus were found such as *Low temperature and salt responsive protein family*, *heat shock protein 70*, *dehydrin family protein*, *late embryogenesis abundant protein (lea) family protein* and multiple *ethylene-responsive transcription factors* (not illustrated).

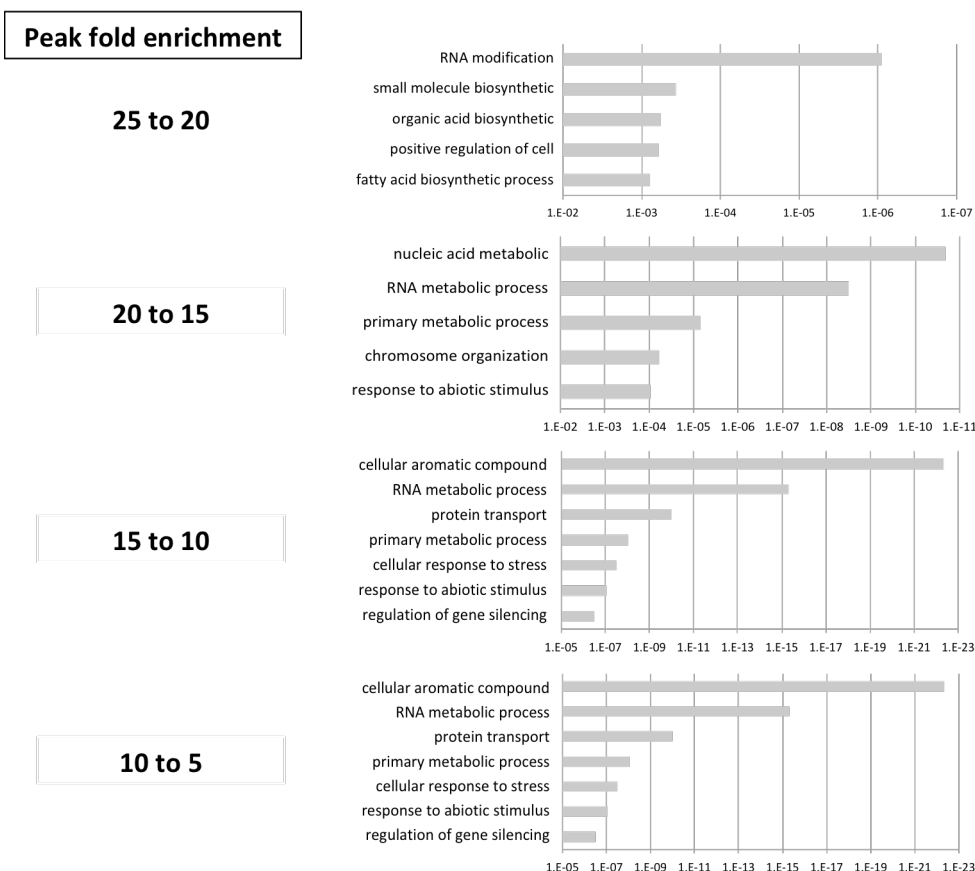


Figure 6.5 | Gene ontology (GO) enrichment for PavDAM5 targeted genes

After analysis, a set of genes were obtained with different fold enrichment. Here is presented GO terms for set of genes with different fold of enrichment (from 25 to 5). The GO term are represented in -log10 (p-value).

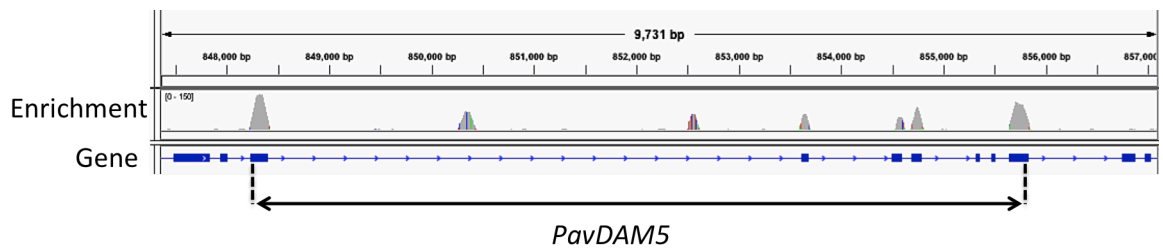


Figure 6.6 | PavDAM5 *in vitro* ChIP-seq profile

IGV (Robinson *et al.*, 2011) screenshot of *in vitro* PavDAM5 enrichment (upper panel) at *PavDAM5* loci. Exons are represented by blue rectangles, with blue arrows indicating gene directionality.

6.4 Discussion

6.4.1 The key role of DAM genes in the regulation of dormancy phases

The study of a DAM genes peach mutant named ‘*evg*’ has reported that the establishment, maintenance and release of bud dormancy are highly dependent on the expression of DAM genes (Rodriguez *et al.*, 1994; Bielenberg *et al.*, 2004, 2008). In peach, *PpeDAM1* and *PpeDAM6* are up-regulated during bud growth cessation following a change from a long to a short-day photoperiod (Jiménez *et al.*, 2010b) and are down regulated during dormancy release in two different peach cultivars (Leida *et al.*, 2010). In sweet cherry, we also found six *DAM* genes (*PavDAM1-6*) and found similar results for *PavDAM6* where it was up-regulated during the precocious bud development in July until reaching a peak in October during endodormancy onset (Figure 6.2). A dominant expression of *PmDAM1* during the warm months was found in flower bud of Japanese apricot (Sasaki *et al.*, 2011; Zhao *et al.*, 2018) associated with bud formation and seasonal elongation cessation. In our study, similar expression of *PavDAM1* was found in flower buds in sweet cherry (Figure 6.2). In addition, *PavDAM3* as well as all other *DAM* genes were already expressed at different levels in July during floral bud organogenesis suggesting that the combination of these *DAM* genes might play a flowering repressor role until buds are exposed to sufficient cold temperatures to overcome dormancy (Figure 6.2). Nonetheless, each *DAM* genes are highly expressed at different dates conferring potential different role in the regulation of dormancy.

In peach, *PpeDAM5* and *PpeDAM6* have been associated with the dormancy status, their expression were shown to be positively regulated under cold temperature exposure and negatively regulated following a dormancy-breaking treatment (hydrogen cyanamide) (Yamane *et al.*, 2011). Sweet cherry *PavDAM6* and *PavDAM5* exhibited a peak of expression in October or November for the three cultivars (Figure 6.2). Following the expression pattern of *DAM* genes, we hypothesise that all *DAM* genes are expressed during flower bud organogenesis to repress flowering-related gene before dormancy. The expression of *PavDAM6* increases until reaching a peak in October allowing to initiate

endodormancy with *PavDAM3* then *PavDAM5* and 2 are expressed when *PavDAM6* expression decreases (Figure 6.2) allowing to maintain endodormancy. The expression peak of *PavDAM5* and *PavDAM2* are lower in ‘Cristobalina, the earlier cultivar, than the two others (Figure 6.2). In addition, their expression decreases earlier and more rapidly in ‘Cristobalina’, thus we can hypothesise that *PavDAM5* and *PavDAM2* play a role in dormancy depth. *PavDAM4* is the last expressed *DAM* genes in the dormancy cycle and its expression peak and decrease are positively correlated with bud break and endodormancy release as reported in peach (Leida *et al.*, 2010) and in Japanese apricot (Sasaki *et al.*, 2011). *PavDAM4* expression is lowly expressed in ‘Cristobalina’ and decreases two months earlier than the two other cultivars (Figure 6.2). The *PavDAM4* expression peaks occur approximately one month apart between the three cultivars and the decrease of expression is positively correlated with the percentage of bud break (dormancy release date at 50% of bud break) allowing us to hypothesise that *PavDAM4* play a determinant role in endodormancy release and bud break

In summary, we hypothesise that the expression combination of *PavDAM* genes during the early bud development in July-August allows to repress flowering before dormancy. The bud growth cessation (i.e. paradormancy) is associated with the up-regulation of *PavDAM6* and 3. The maintenance and the depth of dormancy is related to the up-regulation of *PavDAM5* and 2. The up-regulation of *PavDAM4* maintains a bud dormant state; once *PavDAM4* decreases, buds resume their growth.

6.4.2 *PavDAM6*: transcriptional activator or repressor?

MADS-box genes are involved in a wide range of processes including the formation of flowers, the control of flowering time and the control of vegetative development in *Arabidopsis* and as well as in crop plants (Schilling *et al.*, 2018). In *Arabidopsis*, MADS-box genes involved in flowering such as *APETALA3* (*AP3*), *PISTILLATA* (*PI*), *SEPALLATA1* (*SEP1*), *SEP2*, *SEP3*, *AGAMOUS* exhibit a MADS-box domain and K-box domain in their protein sequence (Yang *et al.*, 2003; Yang and Jack, 2004). MADS-box genes encode transcription factors that share a common dimerisation and DNA-binding domain (MADS-box domain) and MADS-box proteins bind similar sites based on the consensus sequence CC(A/T),GG named CaRG box (Shore and Sharrocks, 1995; Medard and Yanofsky, 2001). The K domain mediates protein-protein interaction (Yang and Jack, 2004). In sweet cherry, *PavDAM6*, 5 and 4 shared these homologies to the *Arabidopsis* MADS-box proteins (Figure 6.1), thereby confirming that sweet cherry *DAM* genes belongs to the SHORT VEGETATIVE PHASE (SVP)/*AGAMOUS*-LIKE 24 (*AGL24*) subfamily of MADS-box genes as found in

multiple perennials such as peach, apple, Japanese apricot (Bielenberg *et al.*, 2008; Yamane 2008 and 2011; Mimida *et al.*, 2015, Kumar *et al.*, 2016; Schilling *et al.*, 2018). PavDAM proteins contain an EAR (Ethylene-responsive element binding factor-associated Amphiphilic Repression) motif, which is associated with transcriptional repression (Figure 6.1). This repressive motif is highly conserved in transcriptional regulators known to function as negative regulators in a broad range of developmental and physiological processes in plants (Kasan, 2006; Kagale and Rozwadowski, 2010). The presence of the EAR-motif on PavDAM seems to suggest that they might be transcriptional repressors during the dormancy cycle. However, we found that PavDAM6 can activate the reporter genes even in the absence of prey (Figure 6.3 A) in a yeast-two-hybrid assay suggesting a potential positive transcriptional activity of PavDAM6. It was further confirmed by a trans repression/activation assay that PavDAM6 fused to the yeast GAL4 transcriptional activator displayed more β -galactosidase activity than control (Figure 6.3) demonstrating that PavDAM6 enhances the transcriptional activation mediated by GAL4.

In Arabidopsis, *AGAMOUS-LIKE 24 (AGL24)* and *SHORT VEGETATIVE PHASE (SVP)* have an opposite effect in the control of flowering time, however phylogenetic analysis showed that these genes are closely related (Parenicová *et al.*, 2003). *AGL24* functions as a promoter of flowering and is gradually activated in the shoot apical meristem during the floral transition (Yu *et al.*, 2002; Michaels *et al.*, 2003). Whereas *SVP* acts as a repressor of flowering and is activated by daylength or temperature (Hartmann *et al.*, 2000). During floral induction to floral determination, a smooth transition between positive feed-forward and negative feedback loops has been shown and includes *AGL24* and *SVP* (Immink *et al.*, 2012; Tao *et al.*, 2012). During early stages of floral development, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* acts along with *AGL24* and *SVP* to inhibit the expression of the floral MADS-domain factor *SEPALLATA3 (SEP3)* in order to prevent the early differentiation of floral meristems (Liu *et al.*, 2009; Gregis *et al.*, 2009; Fernandez *et al.*, 2014). During the transition from vegetative to reproductive growth, the shoot meristem of flowering plants acquires the inflorescence identity to generate flowers and this phase is led by *SOC1*, *AGL24* and *FT* (Liu *et al.*, 2008; Torti and Fornara, 2012). These results demonstrate that member of *AGL24/SVP* are likely to have multiple molecular mechanisms in the regulation of floral development.

In sweet cherry, we demonstrated that PavDAM6 has an EAR motif, related to negative regulators, but we also show that PavDAM6 alone acts as a positive regulator. Taken together, PavDAM6 may be both activator and repressor. We hypothesise that PavDAM6 alone may activate dormancy-related genes to initiate dormancy. The association with unknown partners during this phase is not excluded. We demonstrated that PavDAM6

interacts strongly with PavDAM5 and itself (Figure 6.3), consistently with findings in Japanese apricot (Zhao *et al.*, 2018b). The expression of *PavDAM6* is up-regulated at the beginning of endodormancy and its decrease is associated with an up-regulation of *PavDAM5* expression. We therefore hypothesise that the interaction between PavDAM6 and PavDAM5 might allow the repression of *PavDAM6* expression and also that above a threshold PavDAM6 might interact with itself to repress *PavDAM6* expression as a negative feedback loop (Figure 6.7).

Our transcriptomic results suggest a negative regulation of PavDAM4, 5 and 6 on the expression of *PavFT* during dormancy. However, despite, the key role of AGL24 and FT in positively regulating flowering in Arabidopsis, no interaction was observed between PavDAMs and PavFT in sweet cherry meaning that the action of PavDAMs on flowering is not trigger by physical protein interactions between these three PavDAMs and PavFT (Figure 6.3 A). Nevertheless, Niu *et al.* (2015) demonstrated in pear (*Pyrus pyrifolia*) that some PpDAMs interacted with the promoter of *PpFT2* in order to regulate bud dormancy and release in pear. However, we did not detect any enrichment of PavDAM5 at *PavFT* loci (Supp. Figure 6.1).

6.4.3 PavDAM5, a potential activator of cold response genes

In addition to an interaction between PavDAM5 and 6, PavDAM5 interacts with itself in yeast, nonetheless it remains complicated to conclude about the potential activity of PavDAM5. An *in vitro* ChIP-seq experiment was performed using tagged PavDAM5 proteins to identify potential target gene of PavDAM5. It was shown that targeted genes were enriched in GO terms such as response to abiotic stimulus, RNA process, fatty acid biosynthesis and cellular response to stress (Figure 6.5). The expression peak of PavDAM5 was reported to be in November during deep dormancy (Figure 6.2) and correlated with colder temperatures, which is consistent with targeted gene pathways (Figure 6.5). So PavDAM5 might act as transcriptional activator of cold response genes during endodormancy and the latter ones could repress flowering-related genes. However, PavDAM5 did not show interaction with PavDAM4 in yeast, in addition the decrease in *PavDAM5* expression was associated with an increase in *PavDAM4* expression reported to be highly correlated to dormancy release and bud break (Figure 6.2; Leida *et al.*, 2010; Sasaki *et al.*, 2011). We hypothesise that PavDAM4 alone or with an unknown partner might repress *PavDAM5* expression to release flowering-related genes from the inhibition of PavDAM5 during endodormancy release (Figure 6.7).

Abiotic stress responses are tightly controlled in plants by EAR-repressors (proteins containing EAR motif) and have been implicated in the control of abscisic acid (ABA) cold, salt, drought and oxidative stress responses (Kazan, 2006). *AP2/ERFs* (*Apetala2/Ethylene Response Factor*) family such as *ERF3*, *4* and *7* are known to contain EAR motif and act as active repressors *in vitro* and *in vivo* (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001; Yang *et al.*, 2005; Kagale and Rozwadowski, 2010). We found several *ethylene-responsive transcription factors* genes targeted by PavDAM5 suggesting that potentially PavDAM5 may target *ethylene-responsive transcription factors* genes to activate their expression, then ERF proteins alone or in combination with PavDAM5 would control some specific pathways involved in cold response.

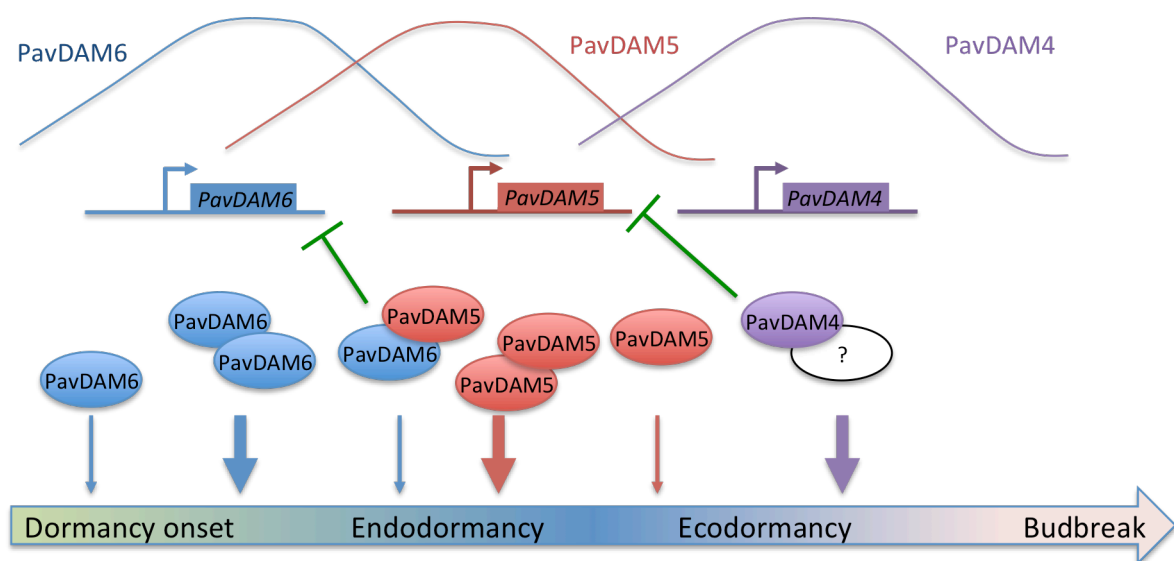


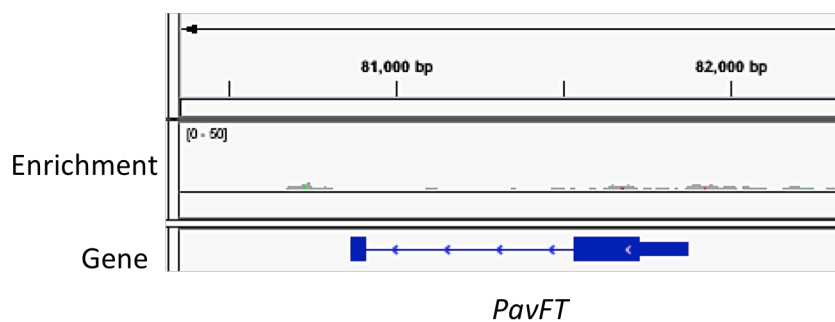
Figure 6.7 | Proposed model of PavDAM interactions and their role in dormancy regulation in sweet cherry
Green bars indicate the negative regulation and arrows the positive regulation.

6.5 Conclusion

Our preliminary study suggested several hypotheses for the regulation and the role *PavDAM* genes involved in dormancy. *In vitro* ChIP-seq experiment should be repeated to remove false positives and in the near future this experiment will be carried out on tagged proteins of PavDAM5 and PavDAM6 to highlight a fine gene regulation of *PavDAM* genes during endodormancy till bud break. In addition, the trans repression/activation assay will be performed on PavDAM5 and PavDAM4 to determine their potential activity as repressor or activator. Taken together these complementary approaches will bring more knowledge about the regulation of dormancy by *DAM* genes.

Additionally, it was shown in Arabidopsis that the repression of *SEP3* by SVP, AGL24, and SOC1 to repress flowering is mediated by recruiting two interacting chromatin regulators, *TERMINAL FLOWER 2/LIKE HETEROCHROMATIN PROTEIN 1* and *SAP18*, a member of *SIN3* histone deacetylase complex (Liu *et al.*, 2009) suggesting potential similar epigenetic regulation through *PavDAMs*. This hypothesis will be further describe in the next chapter.

SUPPLEMENTAL FIGURES



Suppl. Figure 6.1 | PavDAM5 *in vitro* ChIP-seq profile

IGV screenshot of *in vitro* PavDAM5 enrichment (upper panel) at *PavFT* loci. Exons are represented by blue rectangles, with blue arrows indicating gene directionality.

We demonstrated in the Chapter #6 that *DORMANCY ASSOCIATED MADS-BOX* gene expression are linked with dormancy induction, maintenance and release in different sweet cherry cultivars. Furthermore, previous studies revealed that some *DAM* genes are regulated by epigenetic mechanisms such as DNA methylation or histone modifications. To test whether if dormancy is controlled by epigenetic mechanisms, we performed a ChIP-seq analysis for two histone marks (H3K4me3 and H3K27me3) during dormancy. The progress publication is advanced, however more experiments and analysis (such as a large scale ChIP-seq) are needed before submission.

7. H3K4me3 and H3K27me3 modifications are associated with dormancy progression in sweet cherry (*Prunus avium* L.) flower buds

Noémie Vimont^{1,2,3}, Fuxiang Quah³, Mustapha Arkoun², Jean-Claude Yvin², Philip A. Wigge³, Elisabeth Dirlewanger¹, Bénédicte Wenden¹ & Sandra Cortijo³

¹UMR 1332 BFP, INRA, Université de Bordeaux, F-33140 Villenave d'Ornon, France

²Agro Innovation International - Centre Mondial d'Innovation - Groupe Roullier, 35400 St Malo, France

³The Sainsbury Laboratory, University of Cambridge, CB2 1LR, United Kingdom

7.1 Introduction

Seasonal changes require plants to adapt their physiology to survive under unfavourable climatic conditions. Dormancy is a key strategy that allows trees to persist under low winter temperatures and short photoperiods (Faust *et al.*, 1997), enabling the successful reproduction of perennial fruit species including cherry, peach (Yamane *et al.*, 2011; Leida *et al.*, 2012a-b; De la Fuente *et al.*, 2015), pear (Saito *et al.*, 2015) and apple (Mimida *et al.*, 2015). Progress through dormancy is regulated by winter temperatures when buds accumulate chill units until a threshold is met. During this period called endodormancy, bud break is inhibited even under favourable growth conditions (Lang *et al.*, 1987). Following endodormancy release, buds in the second phase of dormancy, namely ecodormancy, are able to resume their growth under the control of warmer temperatures at the end of the winter, leading to bud break and flowering. In the current context of climate change, increasing winter and spring temperatures can affect bud dormancy onset and release in a complex way (Menzel, 2000; Chmielewski and Rötzer, 2001), potentially resulting in lower

fruit production. Despite its importance in fruit production, the molecular events regulating dormancy and its control by temperature are still poorly understood.

Several studies suggest that epigenetic mechanisms are implicated in the regulation of dormancy in perennial species. Recently, Rothkegel and colleagues (2017) showed that DNA methylation and siRNAs participate in the silencing of *PavMADS1* expression during cold accumulation and dormancy release in sweet cherry. *DORMANCY-ASSOCIATED MADS-box (DAM)* genes have been shown to be involved in dormancy in *Rosaceae* (Bielenberg *et al.*, 2004, 2008). Additionally, studies conducted on peach have reported an enrichment of the repressive histone mark H3K27me3 (the tri-methylation of histone 3 at lysine 27) correlated with a decrease in the expression of *DAM* genes during the transition from dormancy to dormancy release (De la Fuente *et al.*, 2015). Conversely, the positive regulation of *PpeDAM6* in dormant peach buds is associated with the presence of the activation histone mark H3K4me3 (the tri-methylation of histone H3 at lysine 4) and H3ac (the acetylation of histone H3) modifications around the transcription start site (Leida *et al.*, 2012b), similarly to results for *DAM1* in leafy spurge (Horvath *et al.*, 2010).

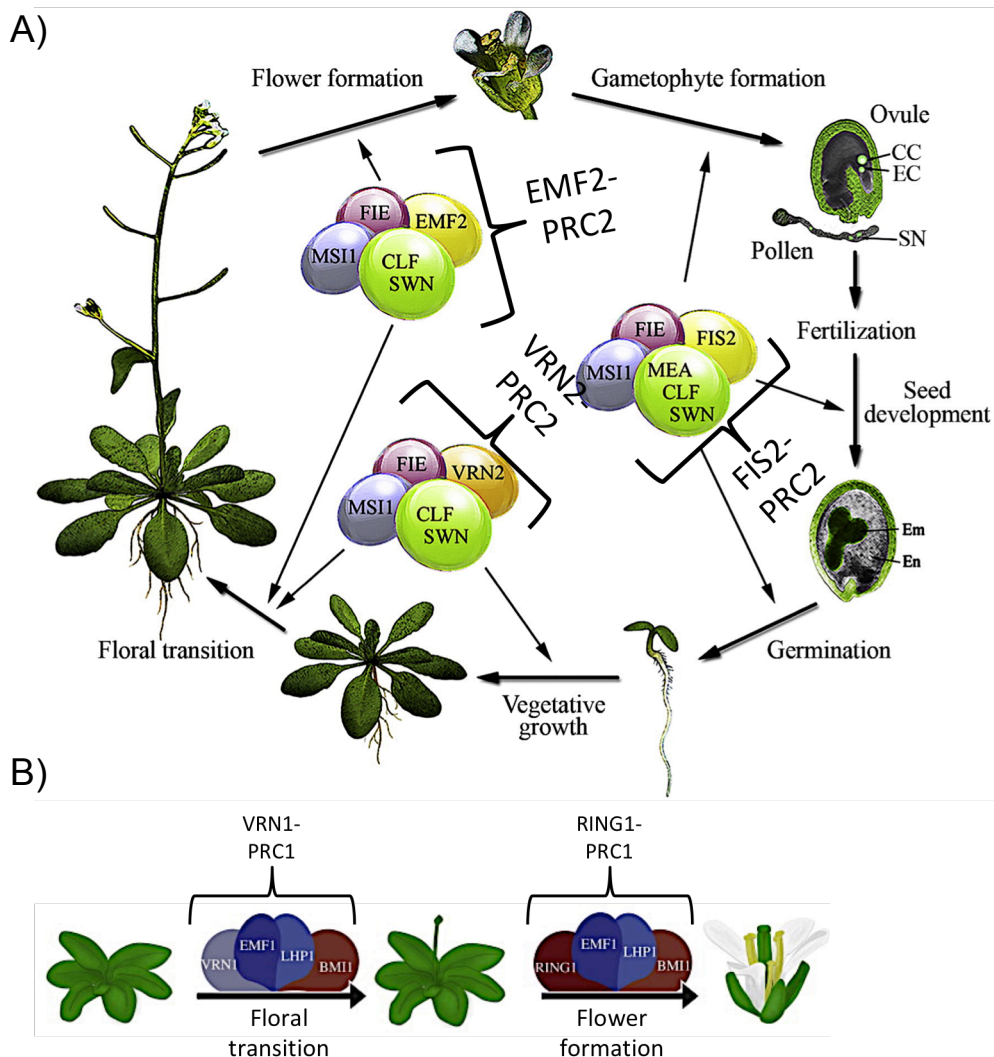


Figure 7.1| Regulation of the Arabidopsis life cycle by the (A) PRC2 complexes and (B) PRC1 complexes. (Butenko and Ohad, 2011; Molitor and Shen, 2013)

In *Arabidopsis thaliana*, it is well documented that H3K27me3 modification is deposited by Polycomb group (PcG) proteins (Goodrich *et al.*, 1997). Polycomb Repressive Complex 1 and 2 (PRC1, PRC2) act in succession to maintain stable gene repression. PRC2 deposits H3K27me3, which is subsequently read by PRC1 and the latter catalyses H2A monoubiquitination (H2Aub1), creating a transcriptionally silent conformation of chromatin (Pfluger and Wagner, 2007; Zheng and Chen, 2011; Molitor and Shen, 2013). PRC2 components are well conserved in plants and the core of the complex is composed of *CURLY LEAF (CLF)*, *SWINGER (SWN)*, *MULTICOPY SUPPRESSOR OF IRA1 (MSI1)* and *FERTILISATION INDEPENDENT ENDOSPERM (FIE)* (Figure 7.1 A). At least three forms of PRC2 exist in *A. thaliana*: (1) the *FIS2 (FERTILISATION INDEPENDENT SEEDS2)* complex associated with *MEDEA (MEA)* controls seed development. (2) the *VERNALISATION2 (VRN2)* complex acts in the vernalisation response and (3) *EMF2 (EMBRYONIC FLOWER2)* complex is active in multiple aspects of vegetative and reproductive development (Figure 7.1 A; Zheng and Chen, 2011; Butenko and Ohad, 2011). The H3K27me3 maintenance is led by PRC1, which is composed of *EMBRYONIC FLOWER1 (EMF1)*, *HETEROCHROMATIN PROTEIN 1 (LHP1)*, also known as *TERMINAL FLOWER2 (TFL2)*, *BMI1* subfamily/*DREB2A-INTERACTING PROTEIN 2 (DRIP2)* and either *VERNALISATION1 (VRN1)* during the floral transition process or *RING1* during flower formation (Figure 7.1 B; Molitor and Shen, 2013).

In this study, we explored the regulation of dormancy by two contrasting histone marks, H3K4me3 and H3K27me3, in the sweet cherry cultivar 'Burlat' during two years of experiments using RNA-seq and ChIP-seq approaches. In addition, we investigated the histone acetylation pathway during dormancy release by the application of exogenous epigenetic drugs.

7.2 Material and Methods

Plant material

1. Winter 2014/2015

Sweet cherry trees (cultivar 'Burlat') were grown in an orchard located at the Fruit Experimental Unit of INRA in Toulence (France, 44°34'N 0°16'W) under standard agricultural practices. Flower buds used for the RNA-seq and ChIP-seq experiment were collected on 21st October 2014 and 5th December 2014 for dormant buds and 27th January 2015 for non-dormant buds. Buds were harvested from the same branches, one branch per replicate with three replicates in total. Upon harvesting, buds were flash frozen in liquid nitrogen and stored at -80°C prior to performing ChIP-seq and RNA-seq.

2. Winter 2015/2016

Samples were collected from 'Burlat' trees grown in an orchard located at the Fruit Experimental Unit of INRA in Bourran, South West of France (48°51'46"N, 2°17'15"E) under standard agricultural practices. Eleven time points spanning the entire period of bud dormancy up to bud break were chosen for RNA-seq. Flower buds were harvested from branches, one branch per replicate from three different trees. Upon harvesting, buds were flash frozen in liquid nitrogen and stored at -80°C prior to performing RNA-seq.

For the exogenous application of epigenetic drugs, branches were collected from the high-chilling sweet cherry cultivar 'Fertard'. Trees were grown in an orchard located at the Fruit Experimental Unit of INRA in Toulence, South West of France (France, 48°51'46"N, 2°17'15"E) under standard agricultural practices. Five branches per modality were randomly harvested from ten trees during four years of experiment when trees had reached three quarter of their chilling requirements.

Measurements of bud break and estimation of the dormancy release date

Details are provided in the 'Material and Methods' section (page 52).

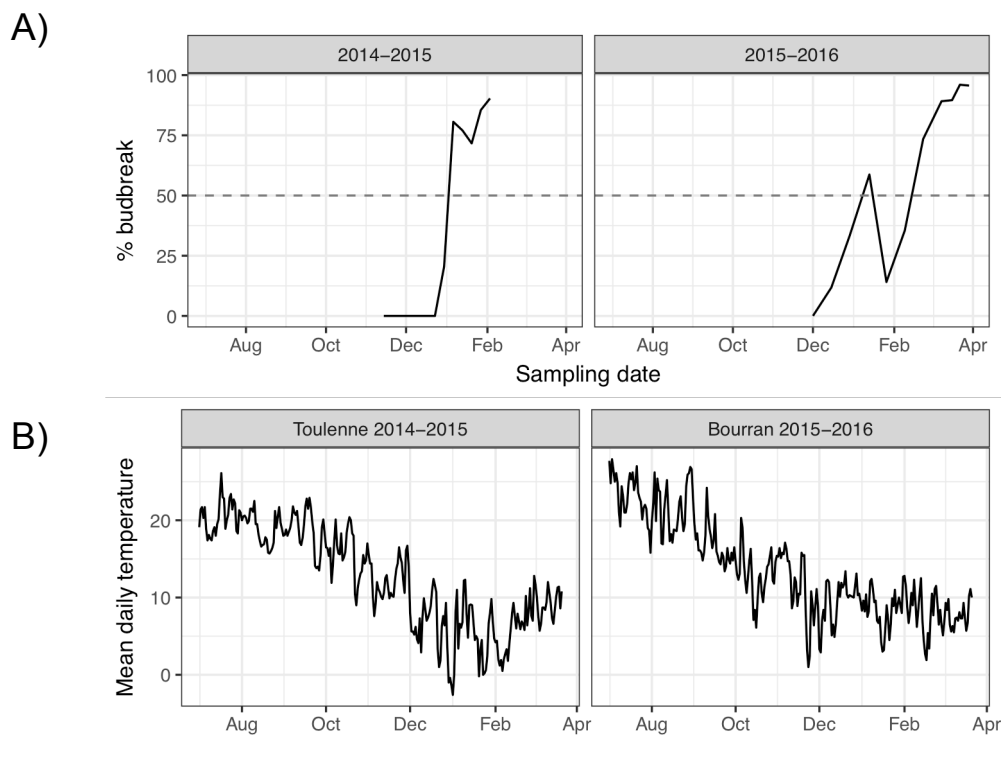


Figure 7.2 | Dormancy status under environmental conditions.

A) Evaluation of bud break percentage under forcing conditions was carried out for the sweet cherry cultivar 'Burlat'. The dotted line corresponds to the dormancy release dates, estimated at 50% of bud break after ten days under forcing conditions. (B) Average daily temperature recorded on site during the sampling season 2014/2015 and 2015/2016.

Treatments with exogenous epigenetic drugs

Details are provided in the 'Material and Methods' section (page 53).

RNA-seq and ChIP-seq

RNA-seq and ChIP-seq were performed on the same biological material from 'Burlat' floral buds in 2014/2015 for a direct and robust comparison of epigenetic regulation and gene expression. Another RNA-seq was performed on the same cultivar in 2015/2016 on eleven time points from bud organogenesis to flowering in order to see the dynamic of gene expression.

1. RNA extraction and library preparation

Details are provided in the 'Material and Methods' section (page 70).

2. RNA-seq data analysis

The raw reads obtained from the sequencing were analysed using several publicly available software and in-house scripts. We first determined the quality of reads using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Possible adaptor contamination and low quality trailing sequences were removed using Trimmomatic (Bolger *et al.*, 2014), before alignment to the peach (*Prunus persica* (L.) Batsch) reference genome v1.0 and v2.0 (Verde *et al.*, 2013 and 2017) using Tophat (Trapnell *et al.*, 2009). Possible optical duplicates resulting from library preparation were removed using the Picard tools (<https://github.com/broadinstitute/picard>). For each gene, raw reads and TPM (Transcripts Per Million) were computed (Wagner *et al.*, 2012). Data were filtered by removing genes that exhibit very small changes in expression (coefficient of variation < 0.3), genes that are lowly expressed (with an average TPM less than 1 across samples) and genes that are not expressed in the majority of samples (with a TPM of 0 for more than three quarters of samples). Differentially expressed genes (DEGs) were assessed using DESeq R Bioconductor package (Anders and Huber, 2010) on filtered data. Genes with an adjusted P-value (p_{adj}) < 0.05 were assigned as DEGs. Z-scores (i.e. (TPM-average)/STD) were calculated from TPM values assigned to DEGs. A Pearson correlation analysis was carried out on DEGs and clustered using heatmap.2 function in the ggplots package of R.

1. ChIP and library preparation

Details are provided in the 'Material and Methods' section (page 64).

2. ChIP-seq data analysis

Sequenced ChIP-seq data were analysed in-house, following the same quality control and pre-processing as in RNA-seq (see Chapter 5). The adaptor-trimmed reads were mapped to

the *Prunus persica* reference genome (Verde *et al.*, 2013) using Bowtie2 (Langmead *et al.*, 2009). Possible optical duplicates were removed using Picard, as described earlier. Averaged profiles of the ChIP-seq signal along the gene body from 500bp upstream to the Transcription Start Site (TSS) to 500bp downstream of the Transcription Termination Site (TTS) were generated using deepTools (Ramírez *et al.*, 2016) for each cluster identified from the RNA-seq data. The ChIP signals for H3, H3K4me3 and H3K27me3 were normalised to the INPUT.

The enrichment visualisation of some genes is represented using the Integrative Genome Viewer (IGV; Robinson *et al.*, 2011) as a tool for visualising sequencing read profiles.

7.3 Results

7.3.1 Identification of two dormancy phases in cherry flower buds: endodormancy and ecodormancy

The two main physiological and developmental phases implicated in perennial dormancy (Lang *et al.*, 1987) were identified in the 'Burlat' cultivar by forcing tests on branches (Figure 7.2 A). The acute increase in bud break percentage under forcing conditions indicates that endodormancy is released and flower buds are able to grow under warmer conditions. Our results show that dormancy release occurs in 'Burlat' on 2nd January 2015 during the 2014/2015 winter, and on 14th February 2016 during the 2015/2016 winter. This one and a half month delay in dormancy release in 2015/2016 is probably due to milder winter temperatures in 2015/2016 (Figure 7.2 B).

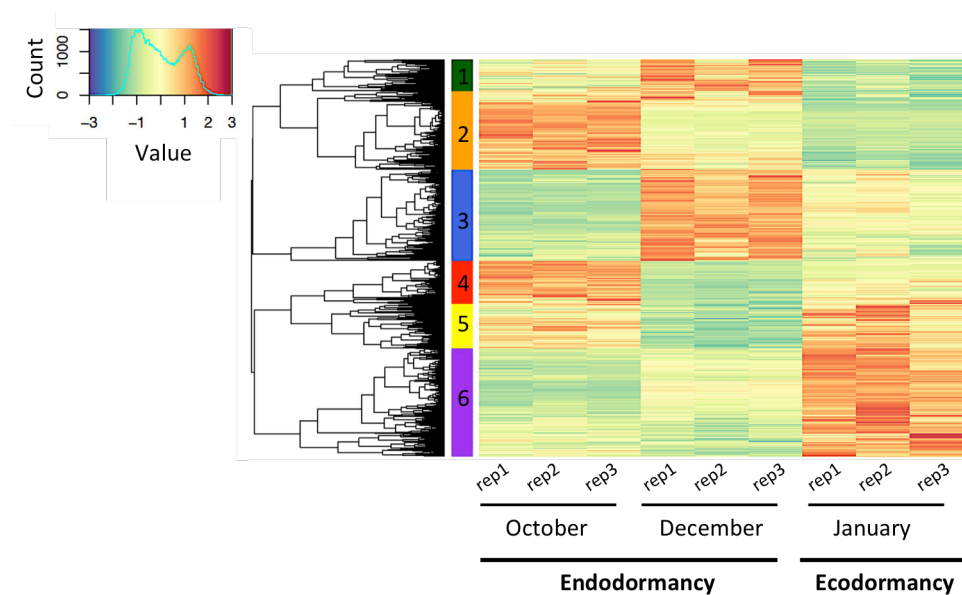


Figure 7.3 |Heat map of differentially expressed genes in October, December and January in 'Burlat' flower buds 2014/2015

The heat map was carried out using Z-score for three different time points (October, December and January) and three replicates for each time point. As represented in the legend, the blue colour represents a relative decrease in gene expression and the orange/red colour indicates a relative increase in gene expression.

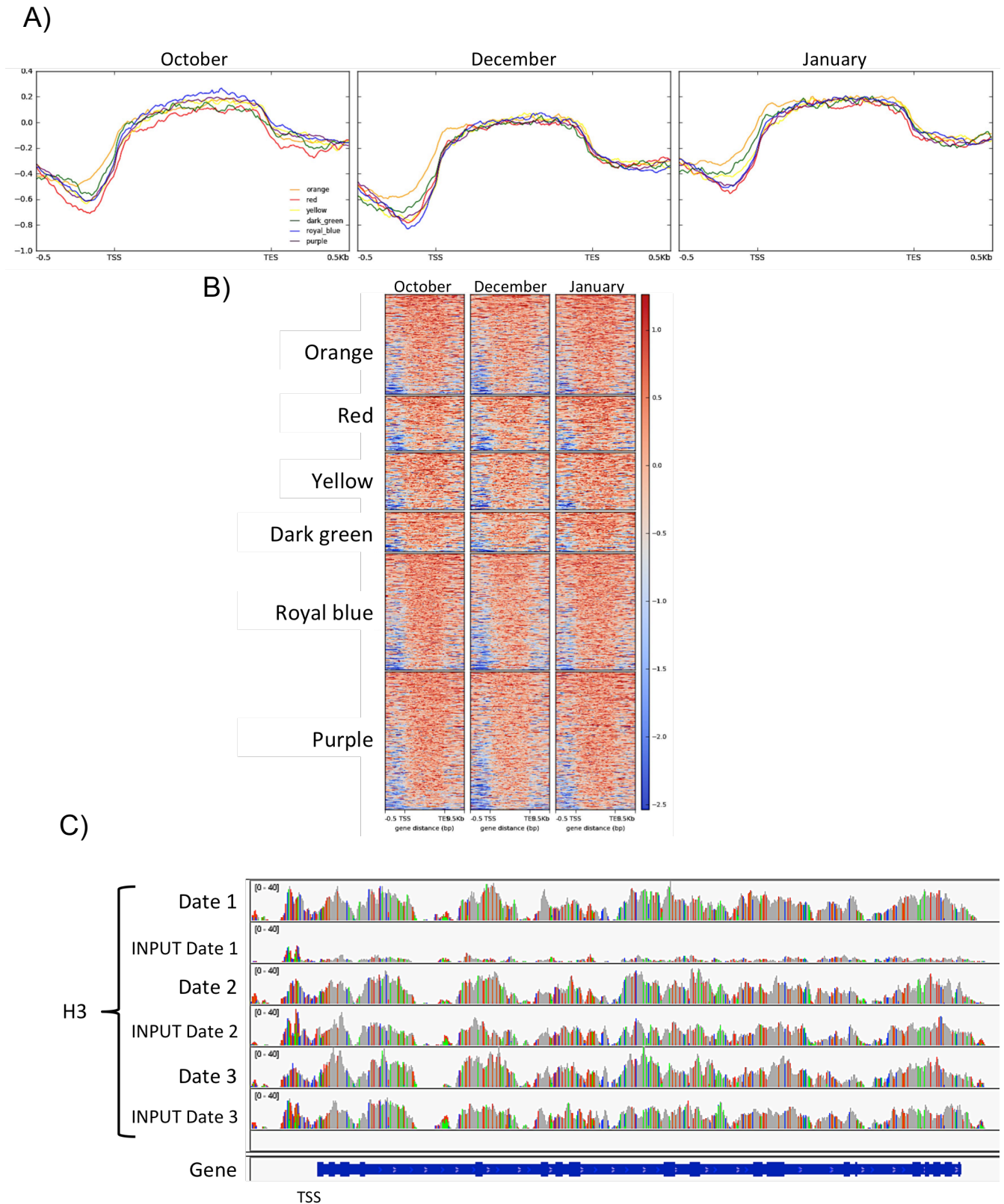


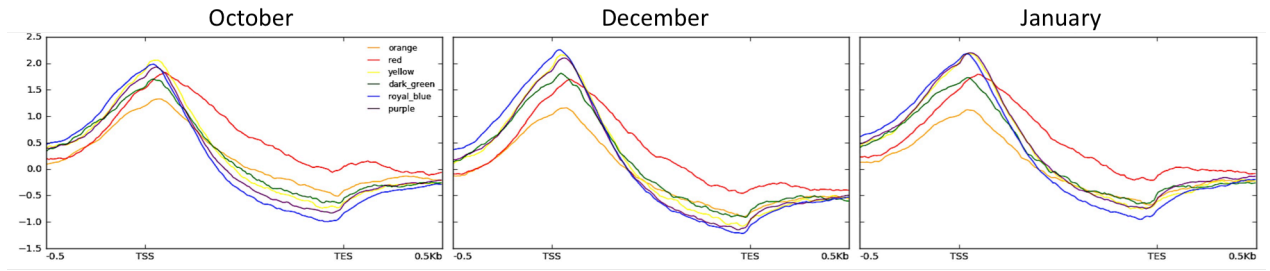
Figure 7.4 | Histone 3 (H3) enrichment during dormancy

(A) Graphic representing the H3 distribution at the gene loci for three different dates and six different clusters. TSS= Transcription Start Site.

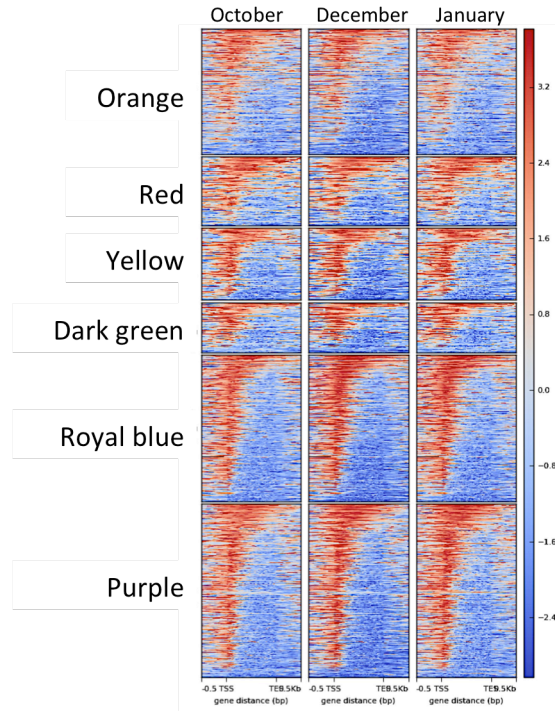
(B) Heatmap of H3 enrichment along six different clusters at three different dates. Red means a high level and blue means a low level for the chromatin marks.

(C) IGV screenshot of ChIP-seq data for H3 performed on 'Burlat' flower buds for three different dates. Genes are represented by blue rectangles, with white arrows indicating gene directionality and taller boxes within the rectangles representing exons.

A)



B)



C)

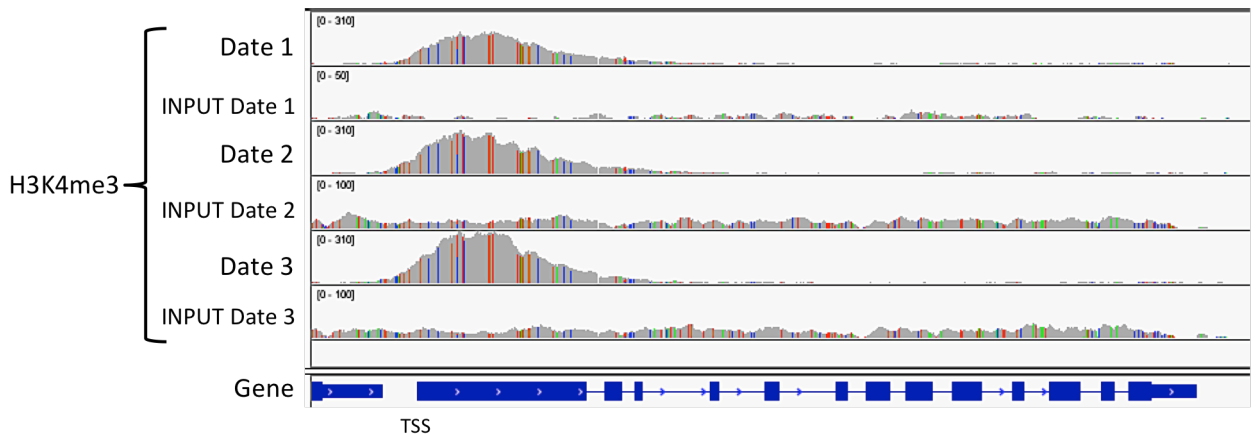


Figure 7.5 | Tri-methylation of histone 3 at lysine 4 (H3K4me3) enrichment during dormancy

(A) Graphic representing the H3K4me3 distribution at the gene loci for three different dates and six different clusters. TSS= Transcription Start Site.

(B) Heatmap of H3K4me3 enrichment along six different clusters at three different dates. Red means a high level and blue means a low level for the chromatin marks.

(C) IGV screenshot of ChIP-seq data for H3K4me3 performed on 'Burlat' flower buds for three different dates. Genes are represented by blue rectangles, with white arrows indicating gene directionality and taller boxes within the rectangles representing exons.

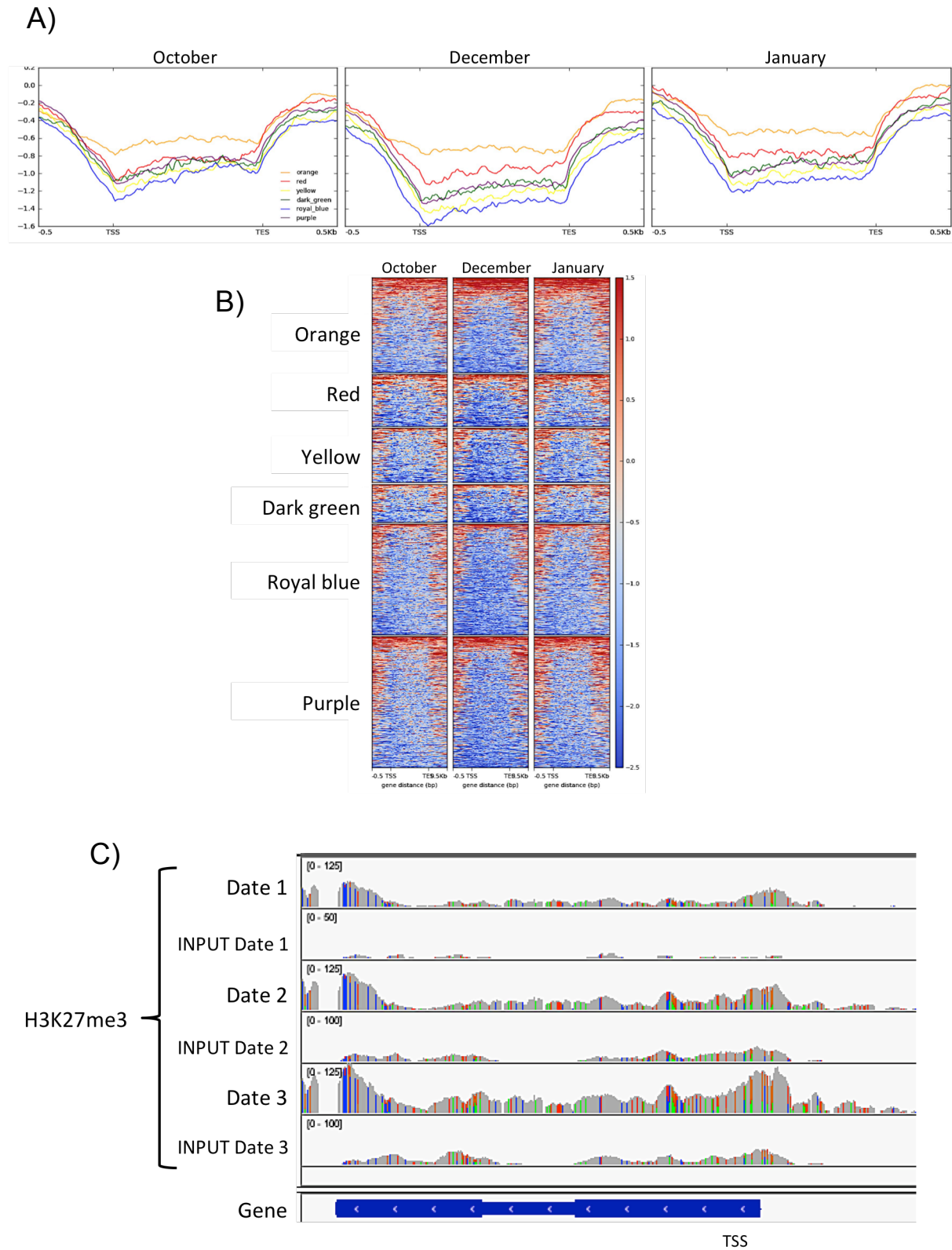


Figure 7.6 | Tri-methylation of histone 3 at lysine 27 (H3K27me3) enrichment during dormancy

(A) Graphic representing the H3K27me3 distribution at the gene loci for three different dates and six different clusters. TSS= Transcription Start Site.

(B) Heatmap of H3K27me3 enrichment along six different clusters at three different dates. Red means a high level and blue means a low level for the chromatin marks.

(C) IGV screenshot of ChIP-seq data for H3K27me3 performed on 'Burlat' flower buds for three different dates. Genes are represented by blue rectangles, with white arrows indicating gene directionality and taller boxes within the rectangles representing exons.

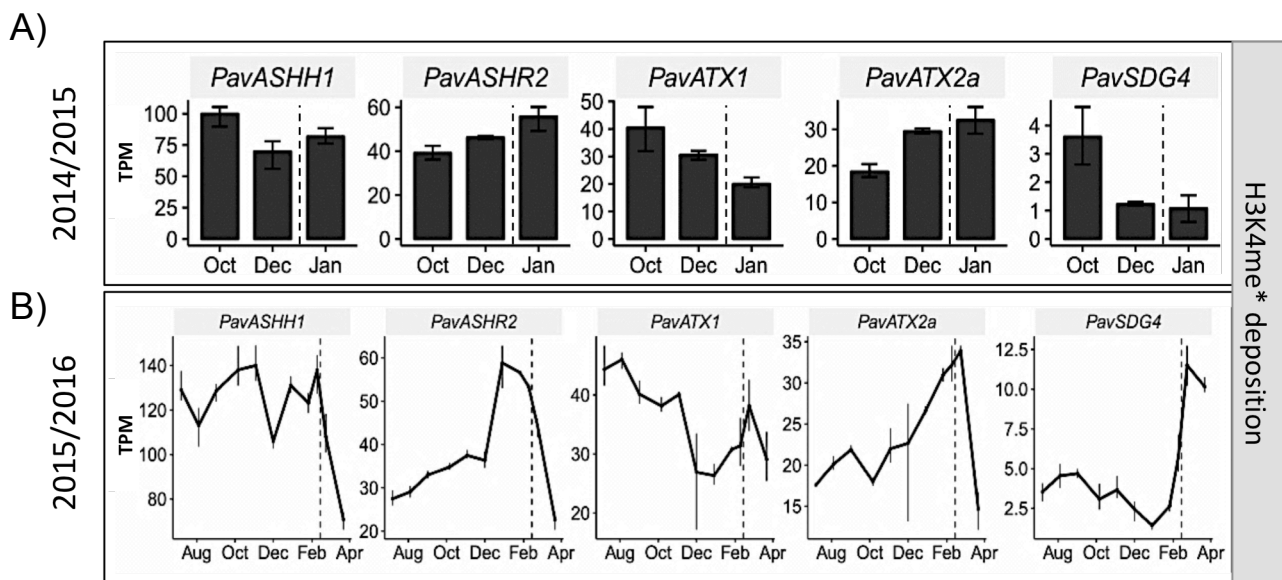


Figure 7.7 | Transcriptional dynamics of genes associated with H3K4me* deposition in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016. Expression of specific genes involved in H3K4me* deposition are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the standard variation between the biological replicates (n=3). Dotted lines represent dormancy release.

7.3.2 Waves of transcriptional activation are observed during endodormancy and ecodormancy

A transcriptomic analysis was carried out to gain an understanding of how the transcriptome changes during the dormancy cycle and how genes related to epigenetic mechanisms are regulated (Figure 7.3).

Differentially expressed genes were clustered based on their expression patterns and the analysis led to the characterisation of six main clusters of gene expression patterns corresponding to groups of genes up-regulated during the different phases of dormancy (endodormancy onset: October; endodormancy: December; ecodormancy: January) (Figure 7.3). Clusters 2 (orange), 4 (red) and 5 (yellow) correspond to genes that are up-regulated in October, therefore corresponding to endodormancy onset. Up-regulated genes (January) are associated with cluster 6 (purple), genes grouped in cluster 5 (yellow) are also upregulated in the ecodormancy phase but their expression is high during dormancy onset as well, suggesting common gene regulation during these two contrasting phases. In contrast, genes up-regulated during deep dormancy (December) are associated with clusters 3 (blue) and 1 (green) (Figure 7.3). These results suggest that physiological and developmental dormancy phases are associated with distinct temporally resolved changes in the transcriptome.

7.3.3 Global changes in H3K4me3 and H3K27me3 during dormancy

We conducted a ChIP-seq for the H3K4me3 and H3K27me3 histone marks, as well as histone 3 (H3) as a control, in order to correlate the abundance of histone marks to the dormancy phases. We characterise their enrichment for each cluster defined based on RNA-seq data (Figure 7.3)

The H3 enrichment along genes is similar between the six clusters for each date and the enrichment was located on the gene body (Figure 7.4 A, B and C; Suppl. Figure 7.1 A and B) as previously reported for this histone mark (van Dijk *et al.*, 2010). These results for the control H3 allowed us to confirm that the ChIP-seq worked and the enrichment differences for the other immunoprecipitations (H3K4me3 and H3K27me3) are not artefacts. However differences in the level of H3 between dates indicate that ChIP-seq data need to be normalised to allow comparisons between dates.

For H3K4me3 histone mark, high enrichment was found at most genes and all dates and a higher enrichment was located at the Transcription Starting Site (TSS) (Figure 7.5 A, B and C; Suppl. Figure 7.2 A and B) as commonly found for this histone mark (van Dijk *et al.*, 2010; Ramírez *et al.*, 2016). Cluster 2 (orange) displayed lower enrichment at the TSS for the three dates compared to the other clusters (Figure 5 A and B; Suppl. Figure 7.2 A and B). Cluster 4 (red) displayed a surprising enrichment of H3K4me3 along the gene body (Figure 7.5 A and B), however analyses of gene length have shown that genes in the red cluster display smaller gene length compared with other clusters (Figure Suppl. 7.4 A) and have large exons (Figure Suppl. 7.4 B). Consequently, the histone mark enrichment was spread along the gene body.

For H3K27me3 histone mark, the enrichment is low and located on the gene body (Figure 7.6 A, B and C; Suppl. Figure 7.3 A and B) as commonly found for this histone mark (Ramírez *et al.*, 2016). In general, the enrichment is very low (Figure 7.6 A; Suppl. Figure 7.3 A) and the majority of genes did not display the histone mark (Figure 6 B). However, the minor number of genes having an enrichment of H3K27me3 displayed a high signal (Figure 7.6 B; Suppl. Figure 7.3 B). The average H3K27me3 signal is higher for the cluster 2 (orange), where a higher number of genes were highly enriched for this repressive histone mark during the three dates (Figure 7.6 A and B; Suppl. Figure 7.3 A and B).

7.3.4 Expression of genes involved in H3K4me* and Polycomb Repressive Complex 1 and 2 (PRC1; PRC2) depositions have distinct patterns during sweet cherry bud dormancy

Prunus avium genes and their *Arabidopsis thaliana* orthologue:
PavASHR2 = ASH1-RELATED PROTEIN 2/SET DOMAIN PROTEIN 39
PavASHH1 = SET DOMAIN GROUP 26

PavSDG4 = SET DOMAIN GROUP 4
PavATX1 = TRITHORAX
PavATX2A = TRITHORAX-LIKE PROTEIN 2
PavCLF = CURLY LEAF
PavSWN = SWINGER
PavEMF2 = EMBRYONIC FLOWER 2
PavVRN2 = VERNALISATION2
PavFIE = FERTILISATION INDEPENDENT ENDOSPERM
PavMSI1 = MULTICOPY SUPPRESSOR OF IRA1
PavMSI2 = MULTICOPY SUPPRESSOR OF IRA2
PavMSI4 = MULTICOPY SUPPRESSOR OF IRA4/ FVE
PavEMF1 = EMBRYONIC FLOWER 1
PavRING 1A = RING 1A
PavDRIP2B-C = DREB2A-INTERACTING PROTEIN 2
PavVRN1D-E-I = VERNALISATION1

To better understand the mechanisms linked to H3K4me3 and H3K27me3 enrichment during dormancy, we investigated expression profiles at the three dates in 'Burlat' cultivar of genes involved in H3K4me* (H3K4me1, H3K4me2 or H3K4me3) deposition and PRC1-2. In addition, we further explored the expression pattern of these same genes in 'Burlat' during another year of experiment spanning the entire bud development. As RNA-seq and ChIP-seq were conducted on the same biological material from 'Burlat' 2014/2015, it is possible to compare the gene expression patterns with the enrichment of histones marks.

We predicted eight genes to be involved in H3K4me* deposition but only five were differentially regulated during dormancy (Figure 7.7). *PavASHR2*, *PavATX2a* and *PavSDG4* were up-regulated just before dormancy release and down-regulated just after dormancy release in 2015/2016 (Figure 7.7 B). *PavASHH1* expression decreased just after dormancy release, while *PavATX1* expression decreased during the time and displayed a slightly increase during dormancy release as well (Figure 7.7 B). Trends of expression found in 2014/2015 were similar to expression patterns observed before dormancy release in 2015/2016 (Figure 7.7 A). These results show that genes involved in H3K4me*, with the exception of *PavSDG4*, are expressed during dormancy and the peak of expression for some of them around dormancy release indicate potential changes in H3K4me* during this period.

We predicted twelve genes linked to the PRC2 complex, which is involved in the deposition of H3K27me3, and eight were differentially regulated throughout flower bud development and dormancy (Figure 7.8). Overall, all genes were upregulated around dormancy release. *PavFIE*, *PavMSI1*, *PavMSI2*, *PavSWN* and *PavVRN2* displayed a peak in expression one month before the dormancy release date for 2015/2016 and sharply decreased afterward (Figure 7.8 B). While the expression peak of *PavCLF*, *PavMSI2* and *PavMSI4* were concomitantly found with the dormancy release date (Figure 7.8 B). However, the RNA-seq time-course resolution in 2014/2015 did not allow us to see the fine regulation

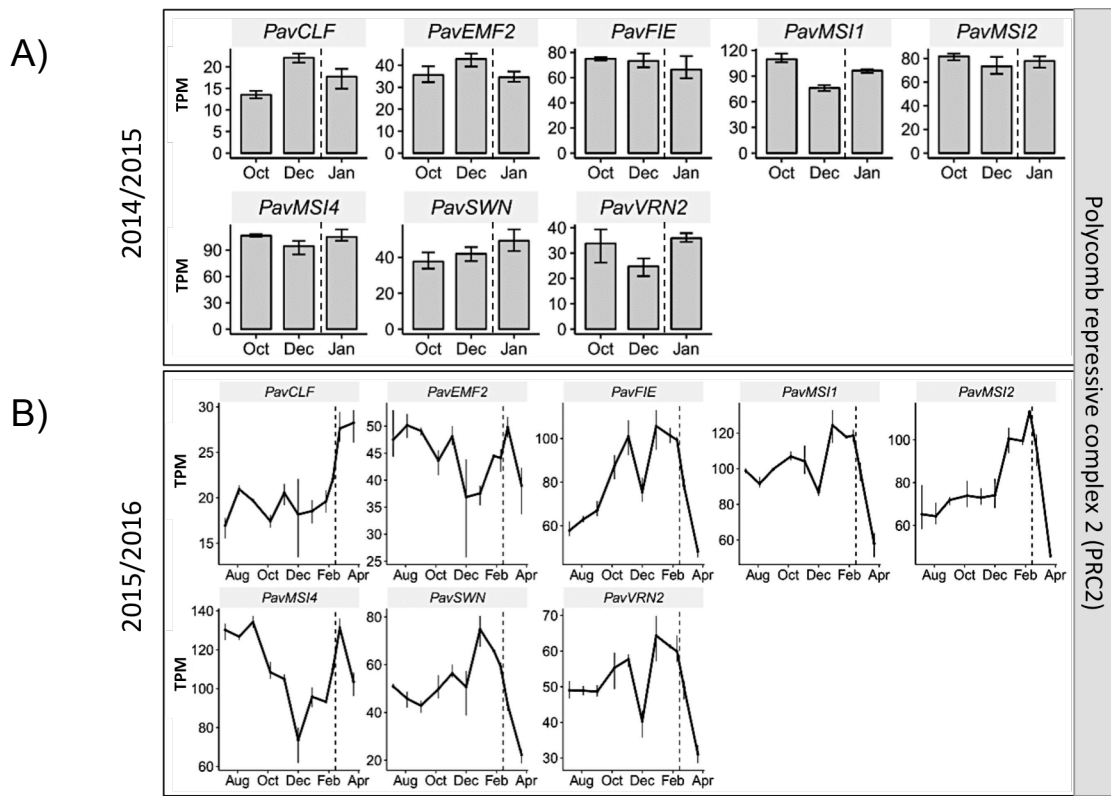


Figure 7.8 | Transcriptional dynamics of genes associated with H3K27m3 deposition in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016. Expression of specific genes involved in Polycomb Repressive Complex 2 (PRC2) associated with H3K27me3 deposition are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the standard variation between the biological replicates (n=3). Dotted lines represent dormancy release.

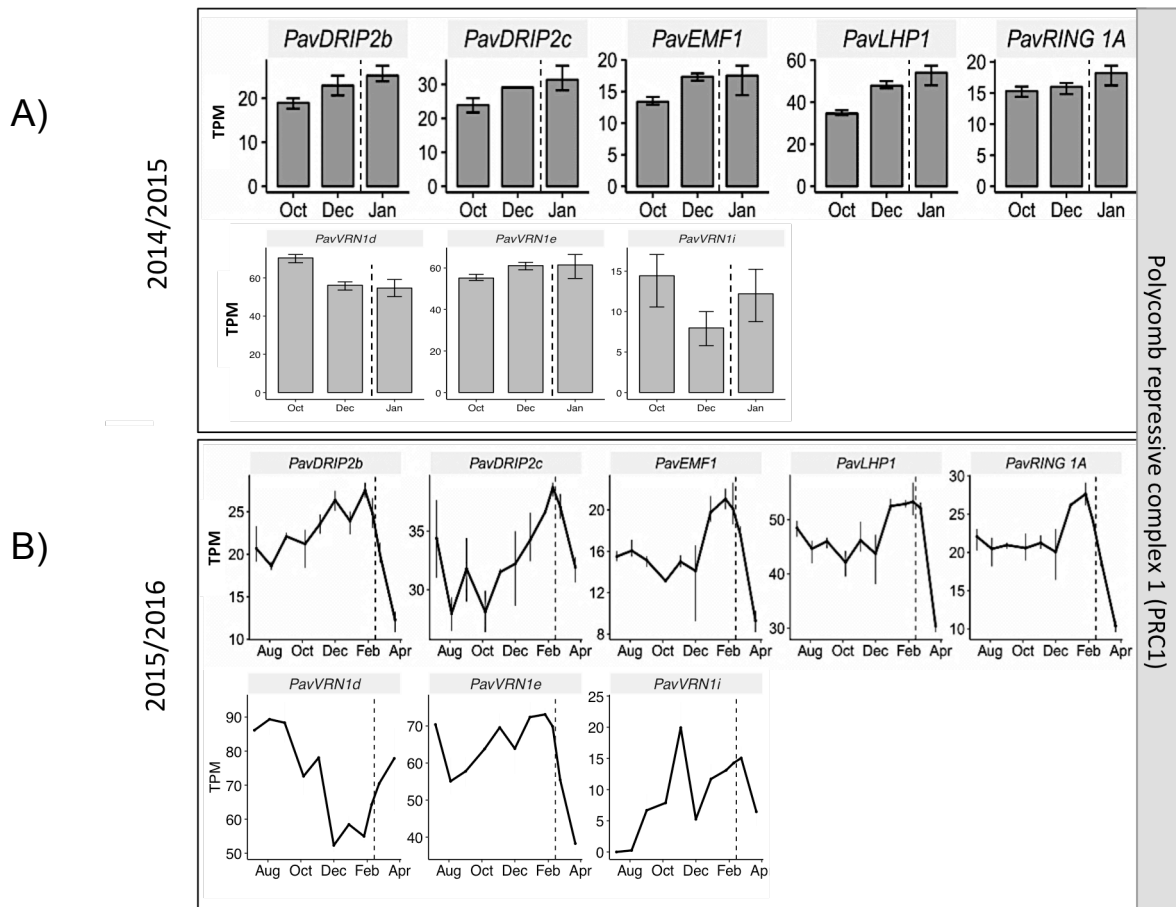


Figure 7.9 | Transcriptional dynamics of genes associated with H3K27m3 maintenance in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016. Expression of specific genes involved in Polycomb Repressive Complex 1 (PRC1) associated with H3K27me3 maintenance are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the standard variation between the biological replicates (n=3). Dotted lines represent dormancy release.

of those genes (Figure 7.8 A).

We identified twelve genes likely to encode components of the PRC1 complex, which plays a role in the maintenance of H3K27me3, and eight were differentially regulated with a marked increase before dormancy release in the 2015/2016 time-course RNA-seq (Figure 7.9 B) as well as in 2014/2015 (Figure 7.9 A). One notable exception is *PavVRN1d*, which is down-regulated upon cold exposure (Figure 7.9). A sharp decrease in expression is observed for all genes after the dormancy release date except for *PavVRN1d* (Figure 7.9 B), however the time-course resolution in 2014/2015 did not allow us to see the expression down-regulation of these genes (Figure 7.9 A). These results show that genes involved in H3K27me3 are expressed at different periods of dormancy and the peak in expression for some of these genes around dormancy release indicate potential changes in H3K27me3 during this period.

7.3.5 Epigenetic drugs involved in histone acetylation/deacetylation impact dormancy release

In order to test whether histone acetylation processes are involved in dormancy release, we tested the effect of exogenous application of histone deacetylase (HDAC) inhibitors and histone acetyltransferase (HAT) inhibitors. Dormancy status was evaluated by the bud break response to forcing conditions (Figure 7.10 and 7.11). The HDAC inhibitors nicotinamide and trichostatin A both delayed dormancy release (Figure 7.10 A), characterised by a lower percentage of bud break after 20 days under forcing conditions compared with the control, which was further confirmed for nicotinamide in 2017 and 2018 (Figure 7.10 B and C). In contrast, the HAT inhibitors curcumin and anacardic acid both accelerated dormancy release, characterised by a higher percentage of bud break (Figure 7.11 A). However, a second experiment in 2018 did not show any acceleration of dormancy release (Figure 7.11 B).

A balance between histone acetylation and deacetylation was found during the dormancy release process

Prunus avium genes and their *Arabidopsis thaliana* orthologue:

PavHAT1 = HISTONE ACETYLTRANSFERASE 1

PavHAC1a = HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 1

PavHAC1b = HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 1

PavHDA6 = HISTONE DEACETYLASE 6

PavHDA3 = HISTONE DEACETYLASE 3

PavHD2C = HISTONE DEACETYLASE 2C

To better understand the mechanisms linked to histone acetylation/deacetylation during dormancy progression, we investigated expression profiles for genes coding for different

Histone deacetylase (HDAC) inhibitors

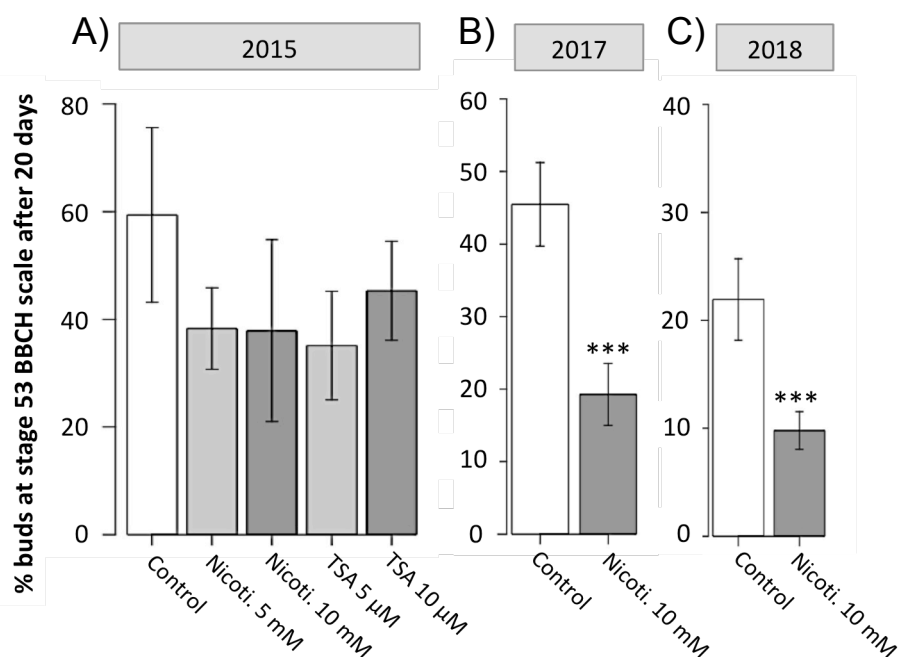


Figure 7.10 | Effect of different Histone Deacetylase (HDAC) inhibitors on the sweet cherry dormancy status. (A) Sweet cherry branches were treated with 5 and 10 mM of nicotinamide (Nicoti.) and with 5 and 10 μ M of trichostatin (TSA) A in 2015, (B) in 2107 and (C) in 2018 and transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded after 20 days. Error bars indicate the data range between the five biological replicates (n=5). Asterisks indicate treatments that differ significantly from untreated branches (Student test, P<0.01:***).

Histone acetyltransferase (HAT) inhibitors

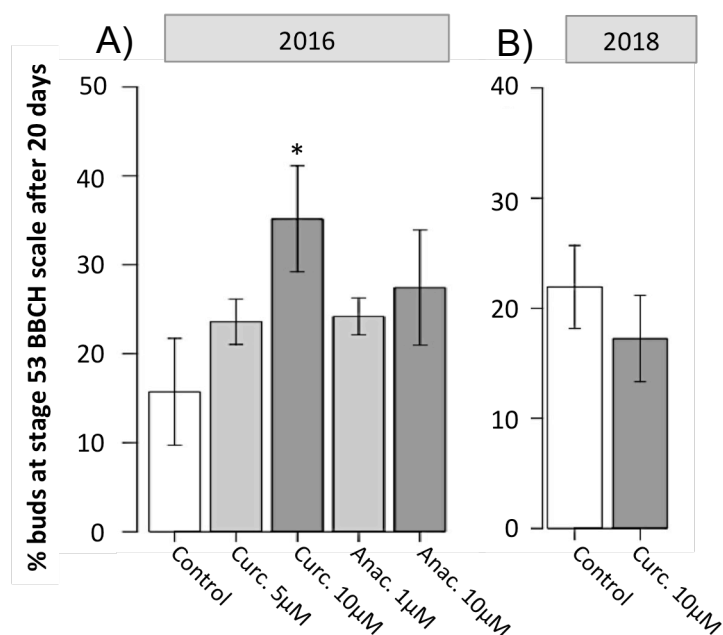


Figure 7.11 | Effect of different Histone Acetyltransferase (HAT) inhibitors on the sweet cherry dormancy status. (A) Sweet cherry branches were treated with 5 and 10 μ M of curcumin (Curc.) and with 1 and 10 μ M of anacardic acid (Anac.) in 2016, (B) in 2108 and transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded after 20 days. Error bars indicate the data range between the five biological replicates (n=5). Asterisks indicate treatments that differ significantly from untreated branches (Student test, p < 0.05).

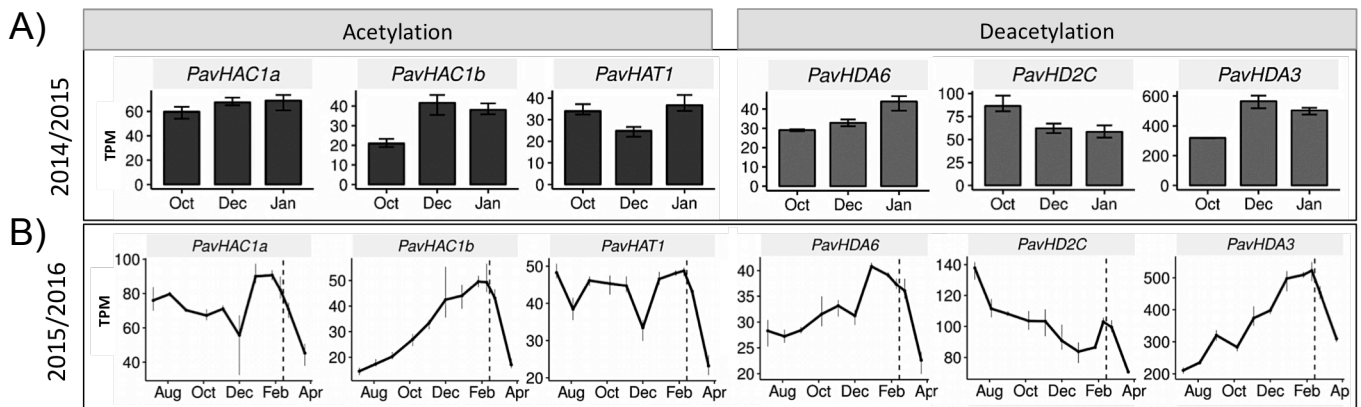


Figure 7.12 | Transcriptional dynamics of genes associated with histone acetylation in the flower buds of 'Burlat' sweet cherry cultivar during dormancy in (A) 2014/2015 and in (B) 2015/2016
 Expression of specific genes involved in histone acetylation are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the standard variation between the biological replicates (n=3). Dotted lines represent dormancy release.

HAT and HDAC (Figure 7.12). Similarities in expression patterns were found between genes encoding HATs or HDACs (Figure 7.12 A and B). *PavHAC1b* and *PavHDA3* expression displayed an increase in expression during dormancy with a peak of expression before dormancy release that subsequently decreases (Figure 7.12 B). *PavHAC1a*, *PavHAT1* and *PavHDA6* expression increased one month before dormancy release and decreased afterwards (Figure 7.12 B). However, a decrease in expression during dormancy was found for *PavHD2C* with a slight increase during dormancy release (Figure 7.12 B). These results show that HAT and HDAC are expressed during dormancy, with peaks observed for both around dormancy release, suggesting that both histone acetylation and deacetylation are happening during dormancy.

7.4 Discussion

7.4.1 The dormancy cycle does not involve global changes in histone acetylation but a fine balance between H3K4me3 and H3K27me3 might impact gene expression

To the best of our knowledge, a global study of H3K4me3 during dormancy has not previously been reported. Studies were mainly focused on epigenetic regulation of specific genes involved in dormancy such as *DAM* genes. In leafy spurge (*Euphorbia esula*), a decrease in H3K4me3 was reported in the promoter of *DAM1* concomitantly with gene repression after prolonged cold exposure (Horvath *et al.*, 2010). Similar results were found in peach (*Prunus persica* L. Batch) for *PpeDAM6* coinciding with the repression of *PpeDAM6* during dormancy release (Leida *et al.*, 2010).

In sweet cherry, our ChIP-seq analysis of the H3K4me3 histone mark, which is associated with transcriptional activation, did not show marked global changes during dormancy, indicating that if H3K4me3 is modified during dormancy, it is for a relatively small number of genes. We found a decrease in H3K4me3 enrichment in cluster 2 (orange) in December, which stays low in January, (Figure 7.5 A and B) associated with the down-regulation of a set of genes during this same period (Figure 7.3). Cell wall, lignin and carbohydrate biogenesis GO term enrichment was highly present in this cluster (Figure Supp. 7.5) with an up-regulation of associated genes in the onset of endodormancy (October) (Figure 7.3). It was demonstrated in sweet cherry that changes in carbohydrate dynamics were linked to changes in dormancy status. More specifically, a degradation of starch into soluble sugars was found during dormancy onset (Kaufmann and Blanke, 2017). In addition, important morphological changes occur during dormancy onset with the biogenesis of dark brown scales to protect sensitive tissues during winter temperatures (Fadón *et al.*, 2015). Taken together, a concomitant decrease in expression and enrichment in H3K4me3 in deep dormancy indicates that the establishment of dormancy by the creation of scales and possible sugar synthesis are associated with H3K4me3 and that the decrease in expression of those genes is associated with a lower H3K4me3 enrichment. Further experiments would be needed to define the potential causality of H3K4me3 in dormancy status. Moreover, the higher enrichment of the repressive histone mark H3K27me3 in cluster 2 (orange) remains stable during dormancy progression (Figure 7.6 A and B). So we hypothesise that genes present in the cluster 2 (orange) are activated in dormancy onset by higher presence of H3K4me3 on the TSS than H3K27me3 on the gene body. However during deep dormancy, the H3K4me3 histone mark is removed, which favours the deposition of H3K27me3 and consequently decreases gene expression. This hypothesis is also reinforced by lower or decreasing expression of four out of five genes (*PavSHR2*, *PavATX1*, *PavATX2a* and *PavSDG4*), which are associated with H3K4me^{*} deposition in endodormancy (Figure 7.7).

Gene activation and silencing also involve histone acetylation and deacetylation. In general, increase in histone acetylation is typically associated with the activation of gene expression, while deacetylation is associated with gene silencing (Kouzarides, 2007; Pfluger and Wagner 2007). In dormant buds of chestnut (*Castanea sativa*), lower histone 4 acetylation (H4ac) levels were observed (Santamaría *et al.*, 2009). In addition to a decrease in H3K4me3 in the region of translation start site of *DAM6* in peach, a decrease in acetylated H3 (H3ac) was observed at the *DAM6* loci (Leida *et al.*, 2010). We show that the application of exogenous HDAC inhibitors on sweet cherry dormant buds during three years of experiments led to a delay in bud break (Figure 7.10), thus suggesting that the maintenance of histone acetylation triggered by HDAC inhibitors at dormancy-related gene loci such as

DAM genes leads to a delay in dormancy release. However, regarding gene expression of HDACs and HATs throughout dormancy (Figure 7.12 A and B), it is not clear whether this change in histone acetylation is essential for dormancy release since simultaneous induction of genes encoding histone acetyltransferases and histone deacetylases was observed (Figure 7.12 A and B). Similar results were reported in hybrid aspen apex (*Populus tremula x tremuloides*; Karlberg *et al.*, 2010). Our results suggest that both histone acetylation and deacetylation are required for the activation or repression of multiple biological processes from dormancy onset to release. However, the epigenetic drug applications have led us to hypothesise that histones at dormancy-related gene loci might be deacetylated to overcome dormancy.

7.4.2 A fine regulation of PRC1-2 seems to be implicated in flower transition and flower formation

Only one study in perennials has reported that the *FIE* gene, a component of the Polycomb Repressive Complex 2 (PRC2), was strongly up-regulated in poplar after a transfer under short-day condition (Ruttink *et al.*, 2007).

We found two *Arabidopsis* PRC2 homologues in sweet cherry involved in the vernalisation pathway and in vegetative and reproductive development: VRN2 complex and EMF2 complex, respectively (Figure 7.8). Interestingly *PavVRN2*, *PavFIE*, *PavMSI1* and *PavSWN*, orthologous of AtVRN2-PRC2, show a concomitant up-regulation one month before dormancy release (Figure 7.8 B), *PavCLF* displayed a later up-regulation (Figure 7.8 B). The AtVERN2-PRC2 complex plays an important role in the initiation of flowering by maintaining repressed *FLOWERING LOCUS C (FLC)*, a floral repressor (Gendall *et al.*, 2001). At present, no FLC orthologues have been found in either the peach genomic sequence or the available sweet cherry transcribed sequences (Castède *et al.*, 2014). We suggest a conserved role for the VRN2-PRC2 complex in sweet cherry to maintain the silencing of a floral repressor still unknown during the floral transition at late endodormancy. In addition, it was demonstrated that the AtVERN2-PRC2 complex interacts with *VERNALISATION INSENTIVE3 (VIN3)*, *VERNALISATION5/VIN3-LIKE PROTEIN (VEL1)* and *VERNALISATION5 (VRN5)* during vernalisation in *A. thaliana* (De Lucia *et al.*, 2008). However, neither *VRN5* nor *VIN3* homologues were found in sweet cherry suggesting that only the core component of the VRN2-PRC2 complex is conserved in sweet cherry but that the newly PRC2 components found in *A. thaliana* may be more specific to the *Brassicaceae* family. Sweet cherry dormancy regulation may be more similar to the regulation of vernalisation in cereals since no homologues of *FLC* or *VIN3* have been found in cereals

(Amasino, 2005), suggesting a possible common mechanism of cold response between perennials and cereals.

In addition to the PavVRN2-PRC2 complex, we found orthologous genes of the AtEMF2-PRC2 complex involved in vegetative and reproductive development, in which *PavCLF*, *PavFIE*, *PavMSI2*, *PavMSI4* and *PavEMF2* display a peak in expression during dormancy release, one month after the expression peak of genes involved in the PavVRN2-PRC2 complex (Figure 7.8 B). It was reported that the AtEMF2-PRC2 complex regulates the transition from the vegetative to reproductive phase by repressing flowering-related gene such as *FT* (Jiang *et al.*, 2008). We suggest a conservation of the EMF2-PRC2 complex in sweet cherry, where the expression peak of EMF2-PCR2 components is found concomitantly with the estimated dormancy release date (Figure 8 B), permitting us to hypothesise that the PavEMF2-PRC2 complex is involved in flower formation as observed in *A. thaliana* (Hennig *et al.*, 2003; Bastow *et al.*, 2004; Jiang *et al.*, 2008). It was suggested that the EMF2-PRC2 complex cooperated with the LHP1 protein (Gaudin *et al.*, 2001; Kotake *et al.*, 2003; Mylne *et al.*, 2006), one component of the Polycomb Repressive Complex 1 (PRC1), which is known to interact with the PRC2 complex to maintain H3K27me3 deposition (Fischle *et al.*, 2003; Molitor and Shen, 2013). In sweet cherry, we identified elements of the PRC1 core complex: *PavDRIP2b*, *PavDRIP2c*, *PavEMF1*, *PavLHP1* are up-regulated during late endodormancy and dormancy release (Figure 7.9 B). Associated with this core complex, *PavVRN1e* and *PavRING1A* were up-regulated during this same period (Figure 7.9 B). Genes encoding the PavVRN2-PRC2 complex were found to be up-regulated one month before those encoding the PavEMF2-PRC2 complex, thereby making it possible to distinguish between these two complexes. In contrary to the PavVRN2-PRC2 complex, genes encoding the PavRING1A-PRC1 and PavVRN1-PRC1 complexes were up-regulated in the period one month before dormancy release up until dormancy release (Figure 7.9 B). This suggests a potential maintenance of the PavPRC2 complexes by both PavRING1A-PRC1 and PavVRN1-PRC1 complexes.

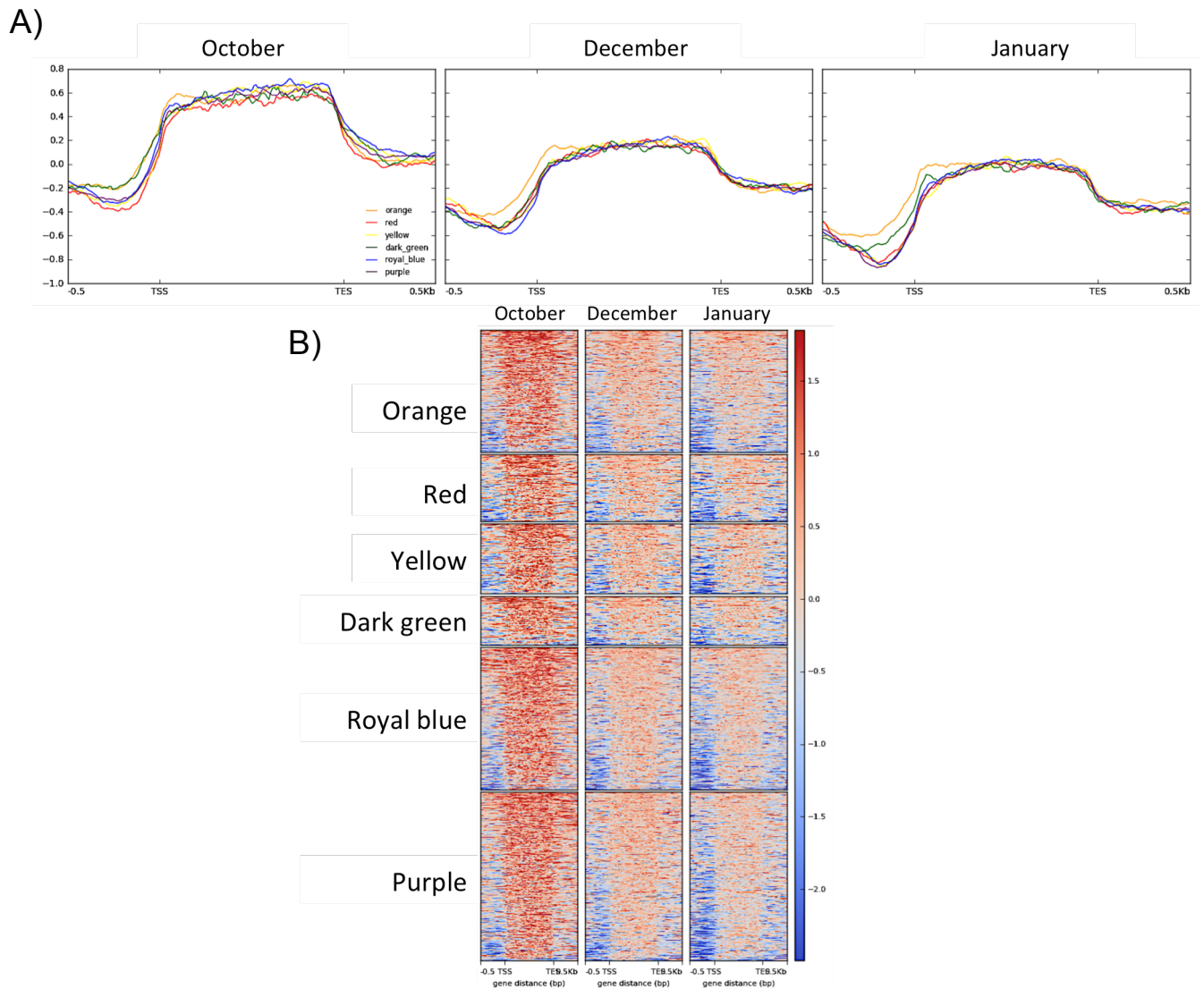
Finally, the expression of all genes involved in PavPRC2 and PavPRC1 complexes are gradually shut down after dormancy release, suggesting a specific implication of PavPRC2 and PavPRC1 complexes in the regulation of the transition from dormancy to flowering in sweet cherry.

7.5 Conclusion

To conclude, the abundance of H3K4me3 found in the specific cluster enriched in GO terms associated with the onset of dormancy, such as carbohydrate and cell wall biogenesis,

seems to indicate that these genes might potentially be regulated by the deposition or removal of H3K4me3. In general, no acute changes were observed for H3K4me3 and H2K27me3 but a more precise study should be carried out to find specific genes enriched by 'peak-calling' analysis. We found that the global H3K27me3 signal was really low but a small number of genes displayed a high enrichment suggesting a specific gene regulation by H3K27me3. We demonstrated that three-time-point resolution chosen during dormancy were not sufficient to see the fine-regulation of gene expression. The exogenous application of epigenetic drugs has confirmed that the bud break regulation is partly led by histone acetylation. In addition, we show that both PRC2 and PRC1 seem to play an important role in the floral transition in floral buds of sweet cherry and also that they act specifically in the regulation of dormancy and mainly during bud break since their expression is shut down after dormancy release.

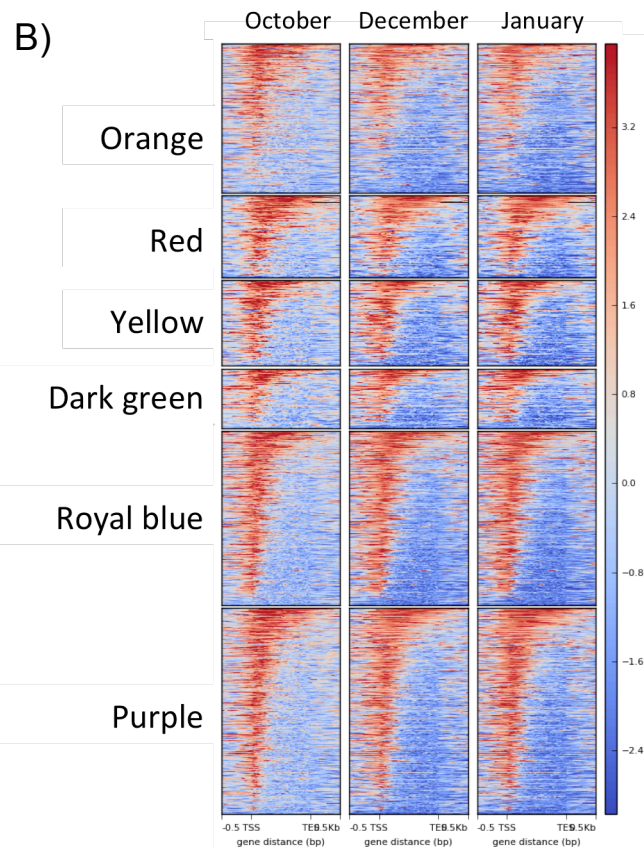
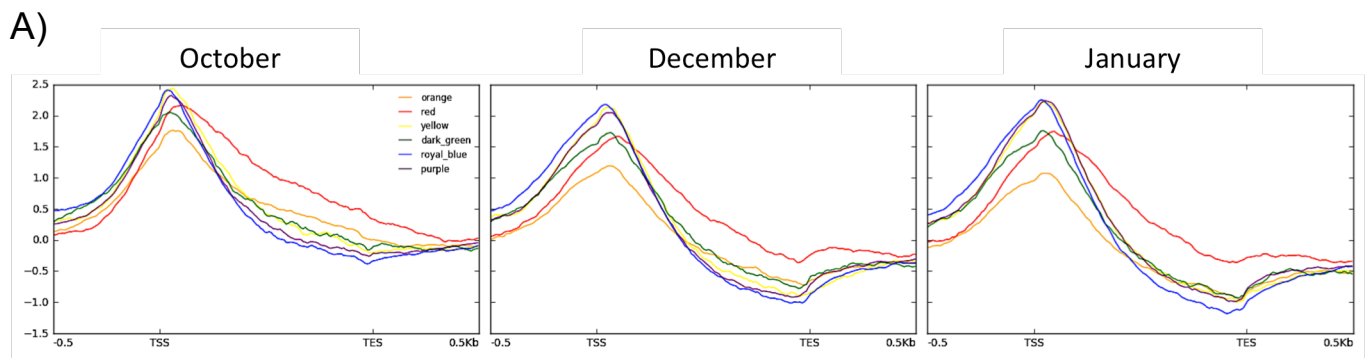
SUPPLEMENTAL FIGURES



Suppl. Figure 7.1 | Histone 3 (H3) enrichment during dormancy replicate 2

(A) Graphic representing the H3 distribution at the gene loci for three different dates and six different clusters. TSS= Transcription Start Site.

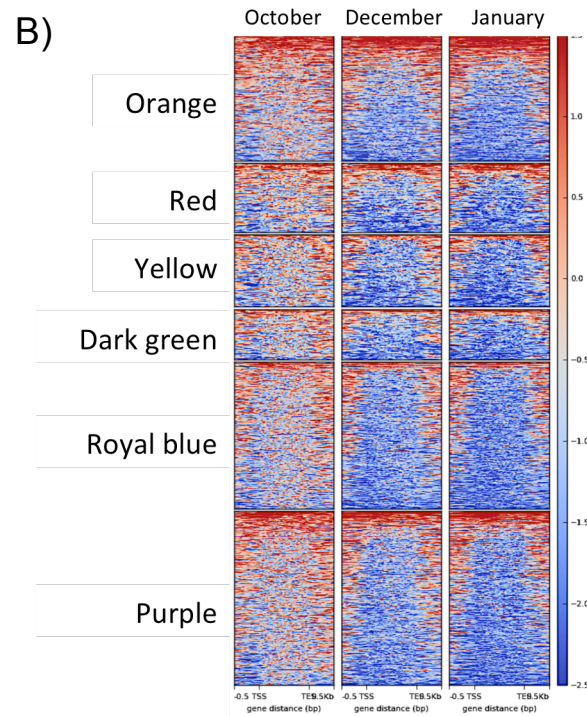
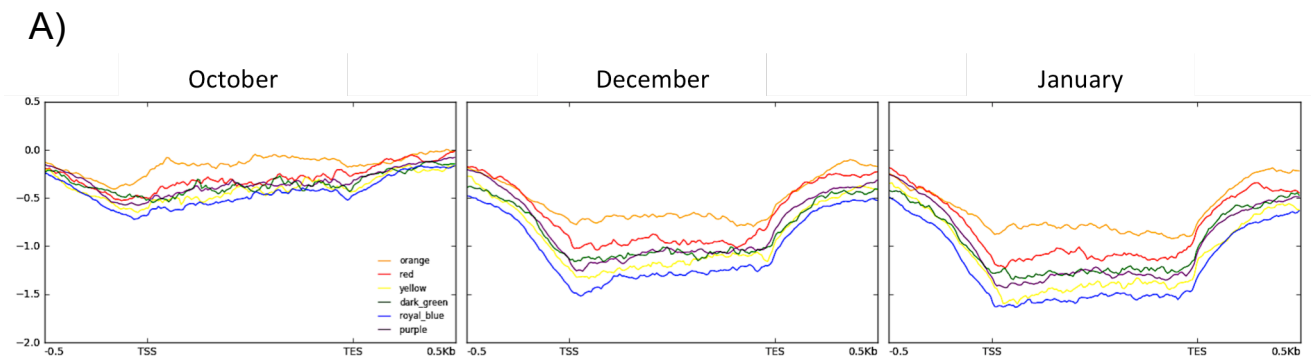
(B) Heatmap of H3 enrichment along six different clusters at three different dates. Red means a high level and blue means a low level for the chromatin marks.



Suppl. Figure 7.2 | Tri-methylation of histone 3 at lysine 4 (H3K4me3) enrichment during dormancy replicate 2

(A) Graphic representing the H3K4me3 distribution at the gene loci for three different dates and six different clusters. TSS= Transcription Start Site.

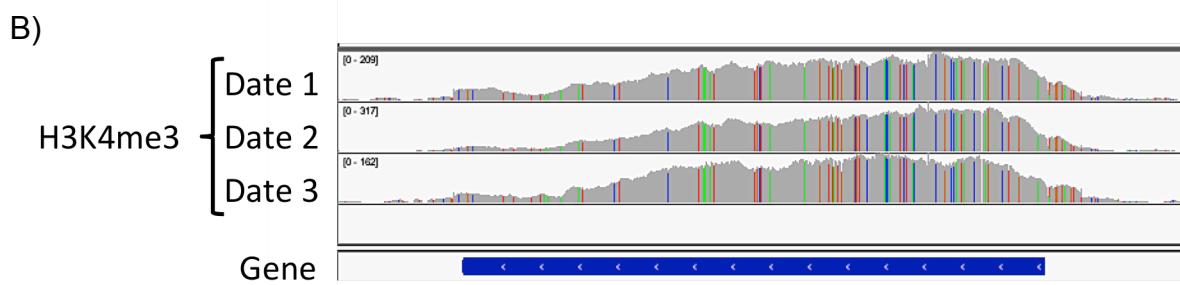
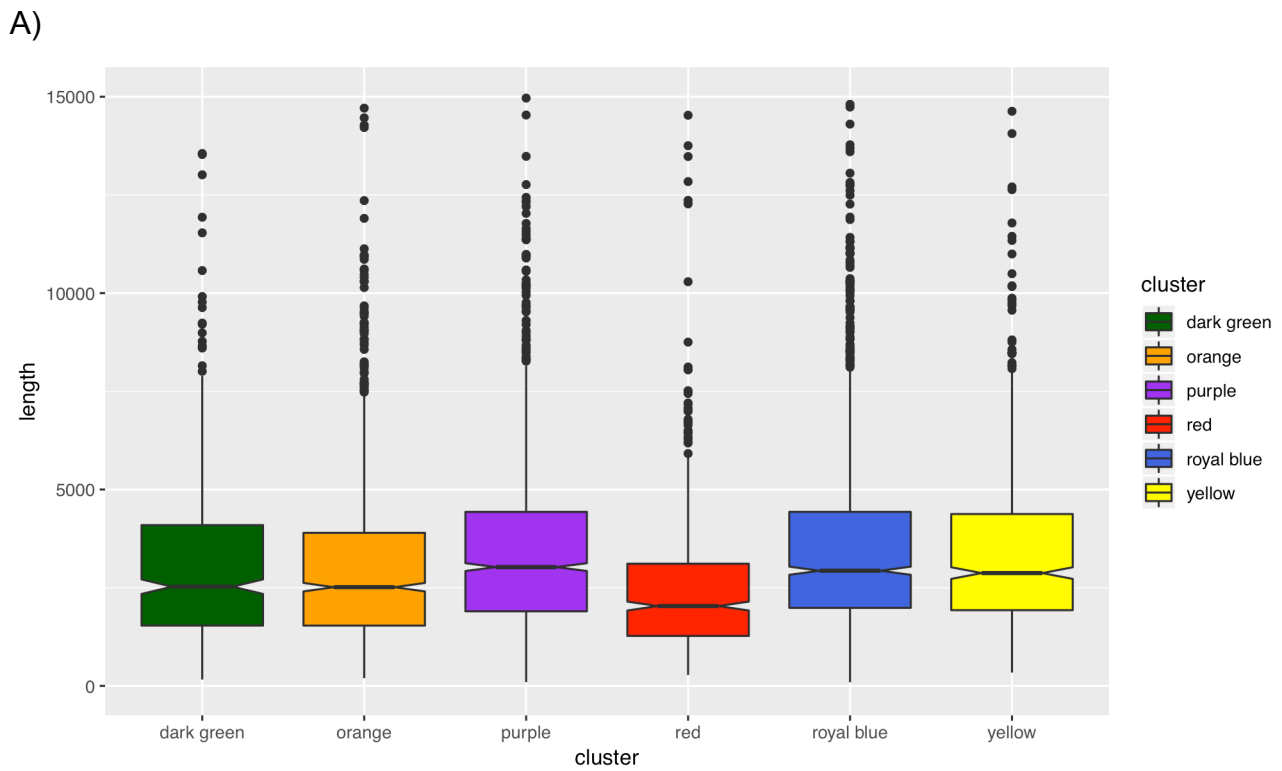
(B) Heatmap of H3K4me3 enrichment along six different clusters at three different dates. Red means a high level and blue means a low level for the chromatin marks.



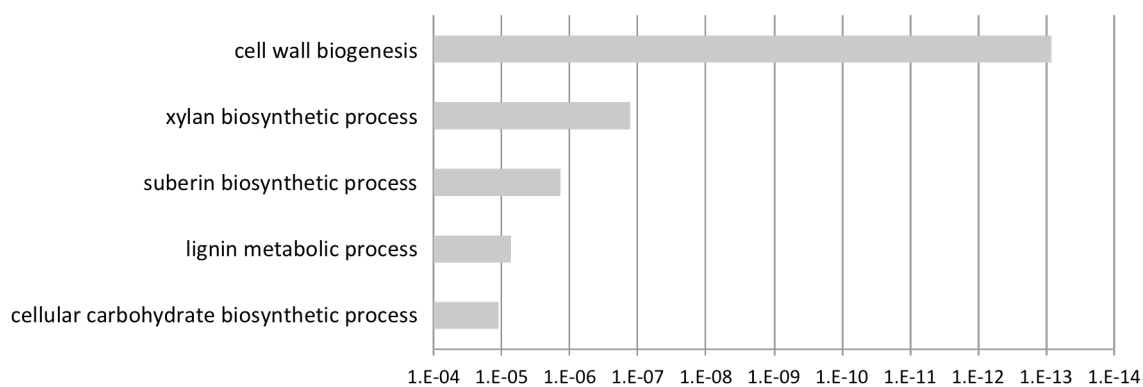
Suppl. Figure 7.3 | Tri-methylation of histone 3 at lysine 27 (H3K27me3) enrichment during dormancy replicate 2

(A) Graphic representing the H3K27me3 distribution at the gene loci for three different dates and six different clusters. TSS= Transcription Start Site.

(B) Heatmap of H3K27me3 enrichment along six different clusters at three different dates. Red means a high level and blue means a low level for the chromatin marks.



Suppl. Figure 7.4 | (A) Gene length per cluster and (B) H3K4me3 ChIP profile of one gene present in the cluster orange.



Suppl. Figure 7.5 | Gene ontology (GO) enrichment for the cluster 2 (orange) for 'Burlat' sweet cherry cultivar in 2014/2015

Data are represented in $-\log_{10}$ (p-value).

We showed in the Chapter #5 that hormone pathways, mainly abscisic acid and gibberellins, changed during bud development. We conducted an analysis of those pathways by transcriptomic and metabolomic approaches to confirm their role in the regulation of bud dormancy. This publication was submitted to eLife journal and is under review.

8. Hormonal balance finely tunes dormancy status in sweet cherry flower buds

Noémie Vimont^{1, 2, 3}, Adrian Schwarzenberg², Mirela Domijan⁴, Rémi Beauvieux¹, Mustapha Arkoun², Jean-Claude Yvin², Sandra Cortijo³, Philip A. Wigge³, Elisabeth Dirlwanger¹, Bénédicte Wenden^{1*}

¹UMR 1332 BFP, INRA, Université de Bordeaux, F-33140 Villenave d'Ornon, France

²Agro Innovation International - Centre Mondial d'Innovation - Groupe Roullier, 35400 St Malo, France

³The Sainsbury Laboratory, University of Cambridge, Cambridge CB2 1LR, United Kingdom

⁴Dept. of Mathematical Sciences, University of Liverpool, Liverpool L69 7ZL, United Kingdom

* **Correspondence:** Corresponding Author: benedicte.wenden@inra.fr

ABSTRACT

In the context of global changes, temperate fruit trees are subject to contradictory effects: longer growing seasons resulting in increased productivity and insufficient chilling during winter leading to flowering abnormalities. It is therefore important to better understand the mechanisms controlling phenology and its response to environmental conditions. Optimal timing and quality of flowering directly depend on adequate dormancy progression during winter and spring, regulated by a combination of chilling and warm temperatures. Physiological, genetic and functional genomic studies have shed light on the mechanisms underlying dormancy control in deciduous trees. Notably, internal signals such as hormones were shown to play a key role in dormancy establishment, maintenance and release. We combined physiological, transcriptional analyses, quantification of abscisic acid (ABA) and gibberellins (GAs), and modelling to further elucidate how these signalling pathways control dormancy progression in the flower buds of two sweet cherry cultivars.

Our results demonstrated that bioactive GAs and associated pathways have distinct functions and may regulate different stages during dormancy. In addition, ABA levels rise at the onset of dormancy, associated with enhanced expression of ABA biosynthesis *PavNCED* genes, and decreased prior to dormancy release. Following the observations that ABA levels are strikingly contrasted between the two cultivars, we showed that dormancy depth is correlated with ABA levels and we identified *PavUG71B6*, a sweet cherry *UDP-Glycosyl*

Transferase gene that up-regulates active catabolism of ABA to ABA-GE in the early cultivar. Subsequently, we successfully modelled ABA content and dormancy behaviour in three cultivars based on the expression of a small set of genes regulating ABA levels. These results underscore the central role of ABA and GA pathways in the control of dormancy progression.

8.1 Introduction

Perennial plants have evolved strategies that enhance survival under the various environmental stresses they face during their growth and reproductive cycles. Among them, dormancy is a quiescent phase that protects meristematic and reproductive tissues from freezing damage. Fruit tree production is highly dependent on the maintenance of viable flower buds through the winter, enabling their rapid emergence in the spring. In temperate trees, the transition from active growth to dormancy is triggered by decreasing photoperiod and temperatures (Heide and Prestrud, 2005; Rohde *et al.*, 2011; Petterle *et al.*, 2013; Singh *et al.*, 2016). As proposed by Lang *et al.* (1987), the bud dormancy process relies on the integration of cold and warm temperatures between endodormancy, when buds are unable to resume growth even under favourable conditions (Rohde and Bhalerao, 2007), and ecodormancy, when bud development is inhibited by unfavourable conditions until optimal growth temperatures and photoperiod are met. However, in the current context of climate change, temperate fruit trees are affected by contradictory effects and shifts in phenological phases are observed: increased productivity in relation with longer growing season and insufficient chilling during winter (Vitasse *et al.*, 2011; Atkinson *et al.*, 2013; Jochner *et al.*, 2013). In-depth knowledge of mechanisms controlling phenology and the response to environment is therefore necessary to assist breeders in the identification of markers for the development of cultivars more suited to future climates. Dormancy progression and control by temperature and photoperiod in perennial plants have been a focus for decades as it is central for their survival but also in the optimization of fruit tree production. Indeed, physiological, genetic and functional genomic studies have shed some light onto the mechanisms underlying dormancy control in deciduous trees and other perennial plants (Cooke *et al.*, 2012; Ríos *et al.*, 2014; Beauvieux *et al.*, 2018).

Among the identified pathways, the major role of hormones in the regulation of bud growth cessation, dormancy and activity resumption has been extensively discussed (e.g. Cooke *et al.*, 2012; Beauvieux *et al.*, 2018). Plant hormones act in an integrative way and more specifically, GA and ABA balance is often involved in the integration of internal and external cues to control plant growth, as it was extensively studied in seed dormancy (Rodríguez-

Gacio *et al.*, 2009; Finkelstein, 2013; Shu *et al.*, 2018): gibberellins (GA) promotes growth, whereas abscisic acid (ABA) promotes dormancy. Seed and bud dormancy show common features, especially in terms of hormonal control (Powell, 1987; Leida *et al.*, 2012a; Wang *et al.*, 2016).

Indeed, multiple physiological and transcriptomic studies have proposed a central role for abscisic acid (ABA) in the repression of bud activity during dormancy. ABA would function as a signal in response to autumn short days and decreasing temperatures to induce dormancy onset (Rohde *et al.*, 2002; Rohde and Bhalerao, 2007; Ruttink *et al.*, 2007; Wang *et al.*, 2016; Tuan *et al.*, 2017; Li *et al.*, 2018; Tylewicz *et al.*, 2018). This hypothesis was further confirmed by the action of ABA application that can accelerate growth cessation and advance the early stages of bud endodormancy (Guak and Fuchigami, 2001; Li *et al.*, 2018). Strong correlation was also shown between ABA and dormancy depth with high ABA levels detected during endodormancy, followed by a decrease in endogenous ABA content during the transition from endodormancy to ecodormancy (Or *et al.*, 2000; Zheng *et al.*, 2015; Wang *et al.*, 2016; Wen *et al.*, 2016; Chmielewski *et al.*, 2017; Li *et al.*, 2018; Zhang *et al.*, 2018). Recent transcriptomic analyses have further defined the involvement of ABA in bud dormancy. ABA metabolism is associated with the carotenoid pathway through several catalytic steps (Figure 8.1 A). ABA levels are closely modulated by a precise balance between biosynthesis and catabolism (Nambara and Marion-Poll, 2005). Studies have revealed that the critical step for ABA content is catalysed by 9-cis epoxycarotenoid dioxygenase (NCED) because its expression is well correlated with endogenous ABA content. On the other hand, ABA is mainly inactivated by ABA 8'-hydroxylase-catalysed conversion to 8'-hydroxy ABA by cytochrome P450 monooxygenases, encoded by *CYP707A* (Nambara and Marion-Poll, 2003). 8'-hydroxy ABA is then spontaneously converted to phaseic acid (PA), which is further catabolised to dihydrophaseic acid DPA (Figure 8.1 A) by a PA reductase (PAR) encoded by *ABA HYPERSENSITIVE2 (ABH2)* (Weng *et al.*, 2016). In addition, esterification of ABA with glucose by UDP-glycosyltransferases (UGT) produces ABA-GE, which can accumulate in vacuoles or apoplast as storage (Dietz *et al.*, 2000). Expression patterns for *NCED* and *CYP707A*, as well as ABA signalling genes, support ABA involvement in bud dormancy induction and maintenance (Bai *et al.*, 2013; Zhong *et al.*, 2013; Zhu *et al.*, 2015; Wang *et al.*, 2016; Khalil-Ur-Rehman *et al.*, 2017; Li *et al.*, 2018; Zhang *et al.*, 2018).

Regarding GAs involvement in dormancy, studies have suggested their role in repressing the induction of dormancy (Junttila and Jensen, 1988; Ruttink *et al.*, 2007; Olsen, 2010; Eriksson *et al.*, 2015; Singh *et al.*, 2016). Subsequently, in agreement with their role in promoting growth, active GA content increases during ecodormancy (Wen *et al.*, 2016; Zhang *et al.*,

2018). Interestingly, GA treatments have a controversial effect on dormancy and bud break as shown in various perennial species. In fact, GA application may even substitute for chilling (Shafer and Monson, 1958; Rinne *et al.*, 2011; Zhuang *et al.*, 2013), or have delaying effects on shoot growth and bud break (Hoad, 1983; Zheng *et al.*, 2018). The application of GA₄ in poplar induced bud break but not GA₃ (Rinne *et al.*, 2011), and exogenous GA treatments have contrasting effects depending on their concentration (Zheng *et al.*, 2018), suggesting distinct gibberellin functions during dormancy. A major rate-limiting step for increasing endogenous levels of GA₁, GA₃, GA₄ and GA₇, the major bioactive GAs, occurs through the conversion of precursors into active GAs by GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) (Figure 8.1 B; Yamaguchi, 2008). GA catabolism is primarily regulated by GA 2-oxidases (GA2ox). Complex regulations control GA homeostasis throughout plant development, mainly based on feedback loops and interactions between GAs, the receptors GA INSENSITIVE DWARF1 (GID1) and DELLAs that directly regulate GA synthesis and deactivation (Zentella *et al.*, 2007). Until recently, most of the knowledge gathered on the behaviour of the GA pathway during dormancy had been obtained in seeds but reports published in the last years have shed some light on GA regulation throughout bud dormancy in perennial plants (Bai *et al.*, 2013; Zhong *et al.*, 2013; Zhu *et al.*, 2015; Wen *et al.*, 2016; Khalil-Ur-Rehman *et al.*, 2017; Zhang *et al.*, 2018; Zheng *et al.*, 2018). Although transcriptomic results for GA2ox, GA3ox and GA20ox vary between studies and therefore suggest complex and distinct functions, general patterns could be identified: expression for GA biosynthesis genes GA20ox and GA3ox decreases during dormancy induction then increases after dormancy release and during ecodormancy while GA deactivation GA2ox genes are up-regulated during endodormancy and inhibited after endodormancy is released.

Bud dormancy is controlled by a complex array of signalling pathways that integrate endogenous and environmental cues towards a rest/growth decision. Effort to synthesise the available knowledge and data into modelling approaches have led to the development of mechanistic phenological models based on the endo- and ecodormancy phases regulation by temperature and photoperiod (Chuine *et al.*, 2016; Chuine and Régnière, 2017). However, no new model for dormancy has been proposed since 1990 (Hänninen, 1990). Current process-based models of bud endodormancy rely on very little information about involved mechanisms mainly because so far, robust molecular or physiological markers of dormancy depth and release are lacking. Functional models for dormancy progression have been proposed based on interactions between respiratory stresses, ethylene and ABA, which in turn activate GA-mediated growth through up-regulation of *FLOWERING LOCUS T* (*FT*) expression and resumption of intercellular transport (Ophir *et al.*, 2009; Rinne *et al.*, 2011). Recently, ABA content has been proposed as a determining factor to assess dormancy

status in sweet cherry (Chmielewski *et al.*, 2017). In this study we sought to integrate a potential hormonal marker of dormancy using a new modelling approach. We have focused on the involvement of GA and ABA pathways in sweet cherry flower bud dormancy. We examined the effect of exogenous GA and ABA on dormancy status and monitored endogenous contents for GAs and ABA and its metabolites, as well as the expression of genes related to ABA and GA metabolism throughout flower bud dormancy for two cultivars with contrasted dormancy release dates. In order to further investigate hormonal control of dormancy, we propose a mathematical model that incorporates the effect of key genes on the dynamics of ABA to estimate dormancy status.

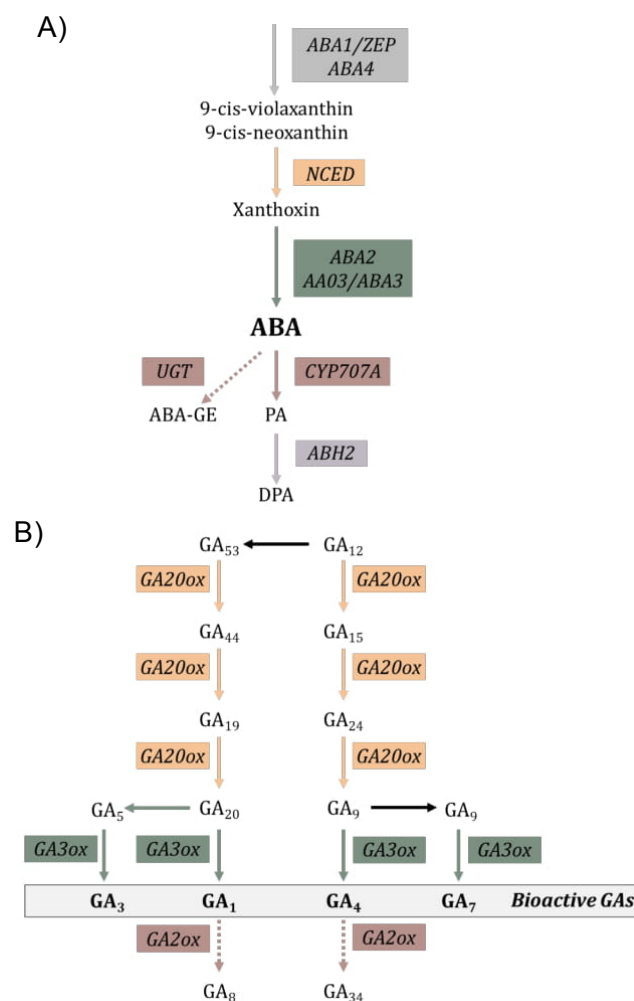


Figure 8.1 | Biosynthesis and catabolism pathway for (A) ABA and (B) GAs.

(A) Abscisic acid (ABA) is synthesised through the action of five enzymes: zeaxanthin epoxidase (ZEP/ABA1), ABA-deficient4 (ABA4,) 9-cis epoxy-carotenoid dioxygenase (NCED), alcohol dehydrogenase (ABA2) and short-chain dehydrogenase/reductase (AAO3/ABA3). ABA catabolism is led by cytochrome P450 monooxygenases (CYP707A) and PA reductase (ABH2, ABA HYPERSENSITIVE2) to convert ABA to phaseic acid (PA) and dihydrophaseic acid (DPA), respectively. ABA can be conjugated with glucose to ABA-glucose ester (ABA-GE) by UDP-glycosyltransferases (UGT). (B) Bioactive GAs (GA₁, GA₃, GA₄ and GA₇) are synthesised by GA 20-oxidases (GA_{20ox}) and GA 3-oxidases (GA_{3ox}) and catabolised by GA 2-oxidases (GA_{2ox}).

8.2 Material and Methods

Plant material

Samples were collected from two different sweet cherry cultivars (*Prunus avium* L.) having contrasted flowering dates. Very low-, low- and high-chilling cultivars (respectively, 'Cristobalina', 'Garnet' and 'Regina') are grown in an orchard located at the Fruit Experimental Unit of INRA in Bourran, South West of France (44° 19' 56" N, 0° 24' 47" E) under standard agricultural practices. Ten time points spanning the entire period of bud dormancy up to bud break were chosen for phytohormone quantification (for 'Cristobalina' and 'Regina') and RNA-seq analysis (for the three cultivars). Flower buds were harvested from branches, one branch per replicate from three ('Cristobalina' and 'Garnet') or two different trees ('Regina'). Upon harvesting, buds were flash frozen in liquid nitrogen and stored at -80°C prior performing RNA-seq and hormone quantifications. Average daily temperatures were recorded by an on-site weather station.

For the exogenous application of hormones, branches were collected from the high-chilling sweet cherry cultivar 'Fertard'. Trees were grown in an orchard located at the Fruit Experimental Unit of INRA in Toulence, South West of France (48° 51' 46" N, 2° 17' 15" E) under standard agricultural practices. Five branches per modality were randomly harvested from ten trees in 2016 and 2018 in mid-January when trees had reached three quarter of their chilling requirements.

Measurements of bud break and estimation of the dormancy release date

Bud break percentage and dormancy status were evaluated using forcing experiments as described in "Materials and methods" section page 52.

Treatments with exogenous hormones and antagonists

To investigate the effects of GA and ABA on dormancy, cherry dormant buds were treated with 5 µM GA₃ (Sigma-Aldrich, ref. 48870), 5 µM GA₄ (Sigma-Aldrich, ref. G7276), 400 µM ABA (Sigma-Aldrich, ref. A1049), 300 µM paclobutrazol (Sigma-Aldrich, ref. 46046), an inhibitor of the GA pathway, and 5 µM fluridone (Sigma-Aldrich, ref. 45511), an inhibitor of the ABA pathway.

All chemicals and a water control were freshly prepared to the desired concentrations in 0.5% of surfactant ("Calanque", Action Pin, Castets, France) to ensure the penetration of active molecules through the bud scales. Chemicals were sprayed on buds under a fume-hood and branches were left several minutes to allow them to dry. Branches were then transferred in the growth chamber (25°C, 16h light/ 8h dark, 60-70% humidity) in pots

containing water. The water was replaced every 3-4 days. Bud break measurements were performed on flower buds as mentioned above.

Phytohormones extraction and quantification

Phytohormone extraction and quantification are described in “Materials and methods” section, page 78.

RNA extraction and library preparation

RNA extraction and library preparation are described in “Materials and methods” section, page 70.

RNA-seq data analysis

The raw reads obtained from the sequencing were analysed using several publicly available software and in-house scripts. The quality of reads was assessed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and possible adaptor contaminations and low quality trailing sequences were removed using Trimmomatic (Bolger *et al.*, 2014). Trimmed reads were mapped to the peach (*Prunus persica* (L.) Batsch) reference genome v2.0 (Verde *et al.*, 2017) using Tophat (Trapnell *et al.*, 2009) and possible optical duplicates were removed using Picard tools (<https://github.com/broadinstitute/picard>). For each gene, raw reads and Transcripts Per Million (TPM) were calculated (Wagner *et al.*, 2012). Data were filtered by removing lowly expressed genes and genes that have a little change in expression (coefficient of variation < 0.3). Differentially expressed genes (DEGs) between time-points were assessed using DEseq2 R Bioconductor package (Anders and Huber, 2010) on filtered data. Genes with an adjusted p-value (p_{adj}) < 0.05 were assigned as DEGs.

Modeling

In order to explore the differences in the expression of ABA in the two cultivars, ‘Cristobalina’ and ‘Regina’, we took a mathematical modelling approach. We constructed a model incorporating information from the genes involved in the ABA signalling pathway.

Since NCEDs and CYP707As and UGT have been implicated in the production and degradation of ABA, respectively, they were considered in the production and decay rates of ABA. ABA level at different times, t , for each cultivar is described by an ordinary differential equation:

$$\frac{d ABA_i(t)}{dt} = p_{NCED1}^i * NCED1_i(t) + p_{NCED3}^i * NCED3_i(t) + p_{NCED4}^i * NCED4_i(t) + p_{NCED5}^i * NCED5_i(t) - \left(p_{CYP707A4a}^i * CYP707A4a_i(t) + p_{CYP707A4b}^i * CYP707A4b_i(t) + p_{UGT}^i * UGT_i(t) \right) * ABA_i(t)$$

for $i=1,2$, where $i=1$ represents the index of cultivar ‘Regina’ and $i=2$ is the index of cultivar ‘Cristobalina’.

In both cultivars, for the sake of simplicity, it was assumed that the rates are linearly dependent on the gene levels. For example, the rate of NCED1–dependent ABA production in Regina at a time t is described by $p_{NCED1}^1 * NCED1_1(t)$ where p_{NCED1}^1 is a non-negative rate constant (model parameter). Genes (NCEDs, CYP707As and UGT) are treated as the time-dependent parameters of the model and their values are taken from the data. More precisely, going back to the earlier example, the level of NCED1 in ‘Regina’, labelled $NCED1_1(t)$, is a function of time t with values calculated from linearly interpolated mean ‘Regina’ data values of NCED1. Initial level of ABA at time 0 in each cultivar, i.e. $ABA(0)$, is taken to be the mean level of ABA on the first day of measurement.

In order to show whether the differential in ABA in the two cultivars could be explained solely by the differences in NCEDs, CYP7074As and UGT, we tested whether there exists a set of parameters where the parameter values for both cultivar models are the same (i.e. $p_{NCED1}^1 = p_{NCED1}^2, p_{NCED3}^1 = p_{NCED3}^2$ and so on) but for which the model simulations can show the ABA differences seen between the two cultivars. Latin Hypercube Sampling was used to select 100,000 parameter sets. Since the gene levels can peak with level (in TPM) of up to 100 times higher than ABA levels (in pg/mg), the production and decay rate constants were bounded above by 0.001 and 0.005, respectively. Once the model solutions were calculated, least squares analysis was performed to calculate the residuals between the models and the mean ABA data of each of the two cultivars. Model with the parameter set that had the lowest sum of the least squares was chosen for simulation and prediction.

Finally, using the mean data measurements for $PavNCEDs$, $PavCYP7074As$ and $PavUGT$ of the cultivar ‘Garnet’ and the model parameter set identified above, we used our model to predict the levels of ABA in the ‘Garnet’ cultivar. Since the initial value of ABA content in ‘Garnet’ cultivar was not measured, we took it arbitrarily to be 1 at the time 0 (this being a value that also falls within the range of the initial ABA levels of the ‘Regina’ and ‘Cristobalina’ cultivars).

8.3 Results

8.3.1 Exogenous GA application accelerates bud dormancy release

To test the effect of exogenous application of hormones on dormancy status, we sprayed dormant branches of the sweet cherry cultivar ‘Fertard’ with solutions containing GA_3 , GA_4 , paclobutrazol, ABA and fluridone. Dormancy status was evaluated by the bud break

response to forcing conditions (Figure 8.2). GA₃ and GA₄ both had significant dormancy alleviating effects, characterised by higher bud break percentage, which was confirmed for GA₃ in a second experiment in 2018 (Figure 8.2 A). However, no antagonist effect, namely bud break inhibition, was observed after a treatment with paclobutrazol.

In both seed and bud dormancy, it is hypothesised that GAs and ABA act antagonistically to control growth resumption and inhibition, respectively. We therefore tested the potential inhibiting effect of ABA on flower bud emergence. We did not observe a significant effect of ABA treatment on dormancy release, but bud break for ABA-treated branches was slightly higher than the control (Figure 8.2 B). Inhibiting ABA biosynthesis with fluridone activated bud break, consistent with the established role of ABA in promoting dormancy.

8.3.2 GA content changes during flower bud dormancy progression

In recent studies, distinct functions were identified for gibberellins during bud dormancy (Zhuang *et al.*, 2013; Zheng *et al.*, 2018). To test whether these results could be confirmed in sweet cherry buds, GA levels were determined over the whole bud development cycle, from flower organogenesis in July to flowering in the spring. We studied two sweet cherry cultivars, 'Cristobalina' and 'Regina', before and during dormancy and their dormancy status was assessed by forcing tests on branches (Figure 8.3). The acute increase in bud break percentage under forcing conditions indicates that endodormancy is released and flower buds are able to grow under optimal conditions. Here, the two cultivars were much contrasted in their dormancy release dates, 'Cristobalina' exhibiting dormancy release on

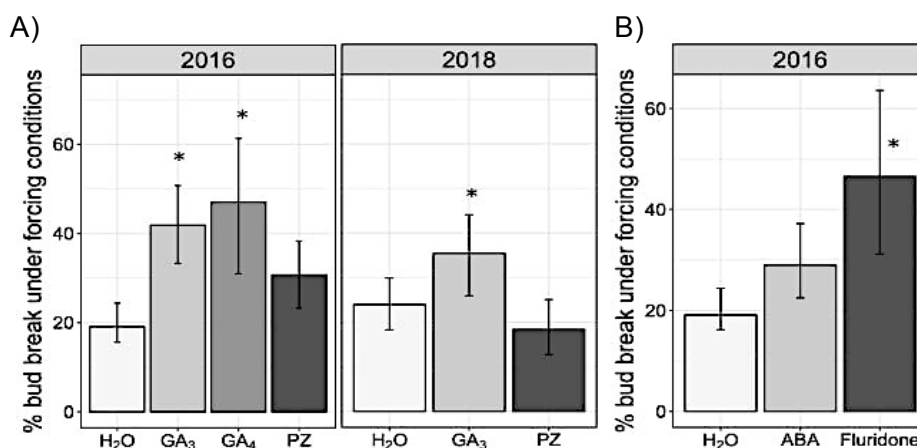


Figure 8.2 | Effect of different GAs, ABA and their inhibitor on the sweet cherry dormancy status. Sweet cherry branches were treated with 5 μ M GA₃, 5 μ M GA₄, 300 μ M, paclobutrazol(GA pathway inhibitor), 400 μ M ABA and 5 μ M fluridone(ABA pathway inhibitor) and transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded after 20 days. Error bars indicate the data range between the five biological replicates (n=5). Asterisks indicate treatments that differ significantly from untreated branches (Kruskal-Wallis test, $p < 0.05$).

December 9th, ten weeks earlier than February 26th for 'Regina'.

First, quantified levels showed large differences between GAs (Figure 8.4). GA₄, GA₇ and GA₉ have a similar pattern over dormancy progression (Figure 8.4) rising from July onwards, which corresponds to flower primordia initiation and organogenesis. GA levels are largely flat during endodormancy, decreasing slightly for the late cultivar and more rapidly for the early cultivar during dormancy release. Content for GA₉, which is the direct precursor of GA₄, was significantly higher than for the bioactive GAs (GA₄ and GA₇) with a concentration reaching 163 and 264 pg/mg in October for 'Cristobalina' and 'Regina', respectively. A notable difference in GA₉ content was observed between these two cultivars during the entire time course, in which the late cultivar buds contained more GA₉ than the early cultivar (Figure 8.4). Among the quantified active GAs, GA₄ was between three and eight times more present than GA₇. GA₄ was the most detected active GA in the early cultivar 'Cristobalina', with a relatively high level reached just after dormancy release in December. By contrast, levels of GA₇ were higher in 'Regina', but with the maximal concentration measured just after dormancy release. In addition, in order to investigate the GA biosynthesis pathway leading to the bioactive GA₁, we measured GA₁₉ content. Interestingly, no GA₁₉ was detectable in the flower buds, except for 'Cristobalina' just before flowering (Figure 8.4).

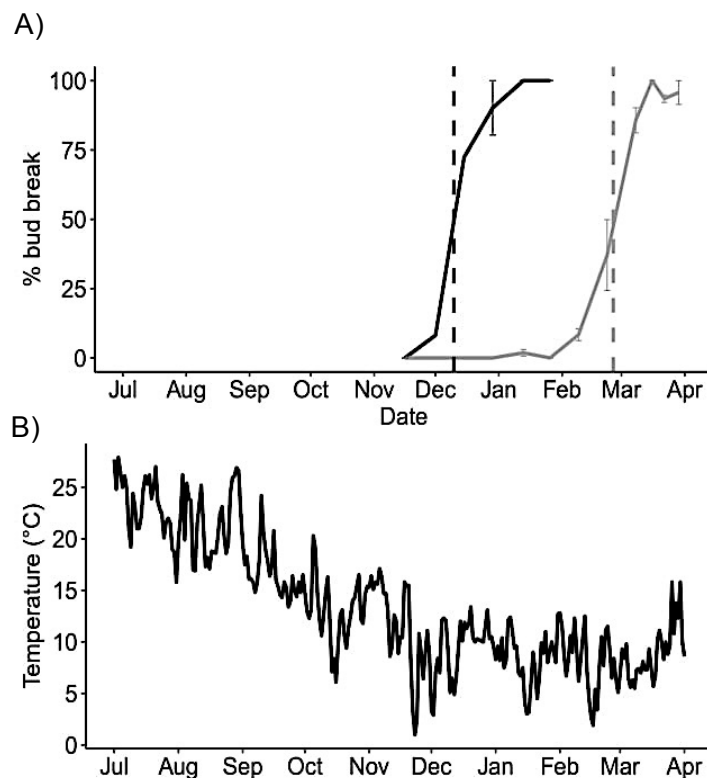


Figure 8.3 | Dormancy status under environmental conditions.

(A) Evaluation of bud break percentage under forcing conditions was carried out for two sweet cherry cultivars displaying different flowering dates. Black: 'Cristobalina', grey: 'Regina' for the early and late cultivar, respectively. The dotted line corresponds to the dormancy release dates, estimated at 50% of bud break after ten days under forcing conditions. Error bars indicate the data range between the biological replicates (n=3 for 'Cristobalina', n=2 for 'Regina'). (B) Average daily temperature recorded on site during the sampling season 2015/2016.

8.3.3 Expression of GA pathway-related genes have distinct patterns during sweet cherry bud dormancy

To better understand the mechanisms linked to the GA pathway during dormancy progression, we investigated genes involved in GA biosynthesis pathway, degradation, signal transduction and response. We predicted seven *PavGA20ox* and five *PavGA3ox* for GA biosynthesis genes (Supplementary Table 9.1) but only three genes were differentially regulated throughout flower bud development and dormancy (Figure 8.5). Interestingly, *PavGA20ox2* displayed two distinct peaks of expression in September and around dormancy release, the first peak rising just prior to a sharp increase in GA₉ levels during dormancy onset. The marked increase in *PavGA20ox1a* expression could be correlated with the production of GA₁₉ after dormancy release (Figures 9.3 and 9.4). The last step of active GA biosynthesis relies on the activity of GA3ox essentially for the production of GA₁ and GA₄.

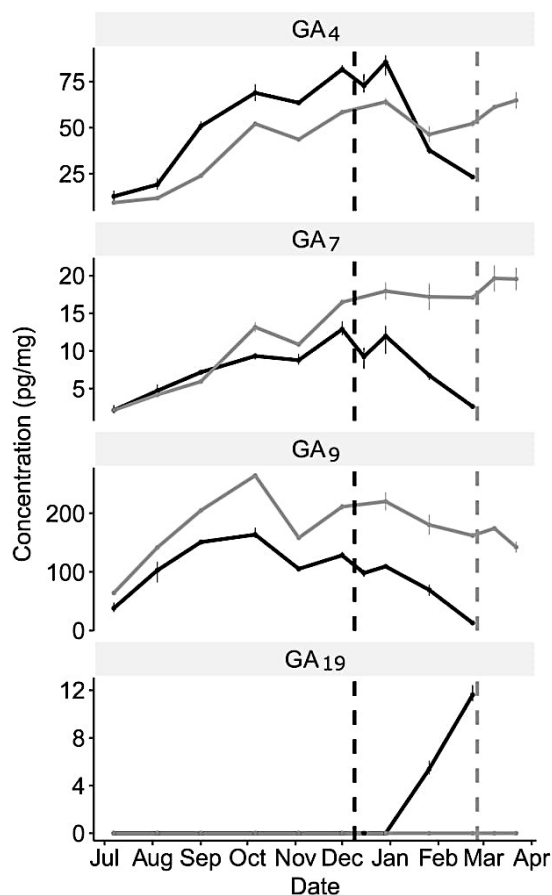


Figure 8.4 | Levels of endogenous bioactive GA₄ and GA₇, and precursors GA₉ and GA₁₉ in the flower buds of two sweet cherry cultivars during bud development.

Black: 'Cristobalina', grey: 'Regina'. Error bars indicate the data range between the biological replicates (n=3 for 'Cristobalina', n=2 for 'Regina'). Dotted lines represent dormancy release.

PavGA3ox1a was the only *GA3ox* transcript detected during dormancy (Figure 8.5) and its expression sharply increases during the early stages of dormancy in October and reaches its highest expression value at maximum dormancy depth in December, for both cultivars. *PavGA3ox1a* is then down regulated after dormancy release, with a marked lag between the two cultivars, potentially linked to their separate dormancy release date (Figure 8.5).

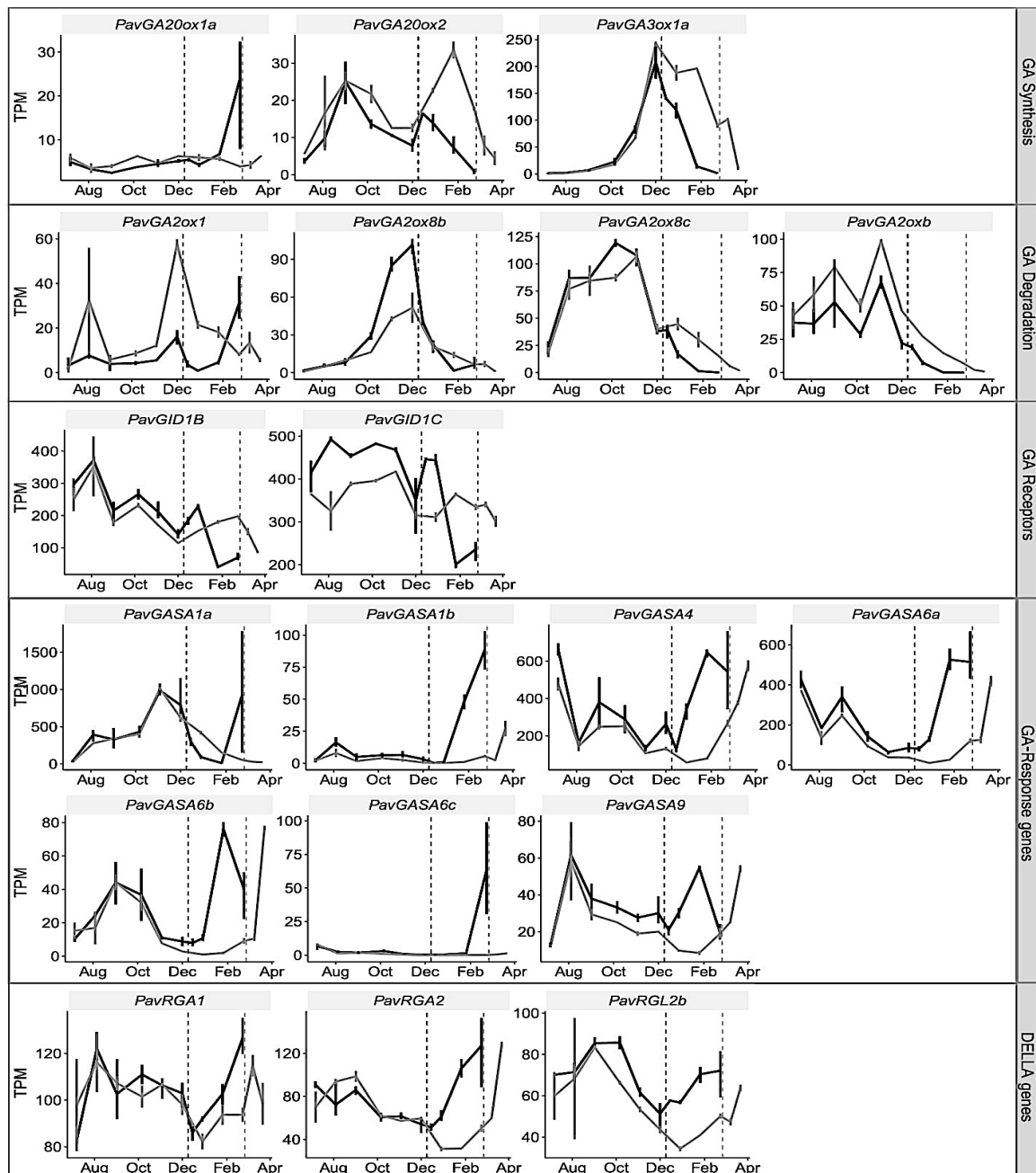


Figure 8.5 | Transcriptional dynamics of genes associated with GA pathway in the flower buds of two sweet cherry cultivars during bud development.

Expression of specific genes involved in GA biosynthesis pathway, degradation, signal transduction and response are represented in TPM (Transcripts Per kilobase Million). Black: 'Cristobalina', grey: 'Regina'. Error bars indicate the standard variation between the biological replicates (n=3 for 'Cristobalina', n=2 for 'Regina'). Dotted lines represent dormancy release.

We then investigated GA receptors and responsive genes (Figure 8.5). Our results indicate that the two identified GA receptor-related genes *PavGID1B* and *PavGID1C* are highly expressed during sweet cherry flower bud development, with a maximum during flower organogenesis (July, August) and early stages of dormancy (September, October). *PavGID1C* expression stays high during dormancy. For 'Cristobalina', expression of both

receptor genes sharply decreased after endodormancy is released. Secondly, we focused on GA-response genes, *GA Stimulated Arabidopsis (GASA)*, that are known to be regulated by GAs (Aubert *et al.*, 1998). Ten *GASA* genes were identified in our transcript dataset and seven were differentially expressed during flower bud development (Figure 8.5). Expression patterns are diverse but for the majority (*PavGASA1b*, 4, 6a, 6b, 6c, 9), a decrease in expression was detected during deep dormancy (November, December), thus suggesting that GA-activated pathways are inhibited during dormancy, despite high contents in GAs (Figure 8.4). However, one notable exception is *PavGASA1a* that is highly activated specifically during dormancy (Figure 8.5).

One way of regulating GA levels is via the catabolism of bioactive GAs. We identified eight *PavGA2ox* that convert GA₁, GA₃, GA₄ and GA₇ into inactive GAs. Four of them displayed differential expression during bud development (Figure 8.5). *PavGA2ox1* and *PavGA2ox8b* were highly expressed during endodormancy, concomitantly with *PavGA3ox1a* expression, thus suggesting a balance between synthesis and degradation that closely controls the levels of bioactive GAs. *PavGA2ox8c* and *PavGA2oxb* were expressed before dormancy and during the early stages of dormancy, in correlation with high contents of GAs and the first peak of *GA20ox2* expression. The expression of *PavGA2ox1* was three times higher for 'Regina' than for 'Cristobalina' while it was the opposite for *PavGA2ox8b*.

The repression of GA by DELLA proteins is well characterised in annuals (Zentella *et al.*, 2007), so to further investigate GA pathway during bud dormancy, we identified ten genes coding for predicted DELLAs, namely *PavGAI*, *PavGAI1*, *PavRGA1*, *PavRGA2*, *PavRGL1a*, *b*, *c* and *PavRGL2a*, *b*, *c* (Supplementary Table 9.1). Three DELLA genes, *PavRGA1*, *PavRGA2* and *PavRGL2b*, were differentially expressed during dormancy (Figure 8.5). Their expression pattern was similar over time and in both cultivars, with an enhanced expression during flower primordia initiation and organogenesis (July to September), a down-regulation during dormancy onset and endodormancy, then up-regulation after endodormancy release.

8.3.4 ABA levels rise at the onset of dormancy

Several studies have highlighted a strong correlation between ABA content and dormancy status. To address this issue in sweet cherry flower buds, we measured ABA levels in both cultivars throughout the flower bud cycle from July to the end of endodormancy (February-March). Results confirmed an increase in ABA content during the early stages of dormancy when photoperiod and temperatures decrease (Figure 8.6). In 'Regina', ABA levels are consistent with the dormancy status exhibiting the highest values during the deepest stages of dormancy (December) and a marked decrease under winter chilling conditions. The

dormancy release phase, illustrated by an increasing bud break percentage under forcing conditions (Figure 8.3) is characterised by decreasing values of ABA content. ABA levels detected during dormancy for the early cultivar ‘Cristobalina’ are much lower than for the late cultivar, associated with an earlier dormancy release date. We can therefore hypothesise that dormancy depth is highly correlated with ABA contents.

To further examine the ABA pathway, levels of PA and DPA, which are catabolites of ABA, were monitored during flower bud dormancy (Figure 8.6). PA and DPA contents follow a similar pattern as ABA but with a month delay approximately between each, and PA and DPA levels are much lower in ‘Cristobalina’ than in ‘Regina’. Esterification of ABA with glucose was also monitored and the concentrations of ABA-GE were higher in both cultivars compared with ABA and its conjugates. The ABA-GE content was constant in ‘Regina’ over the whole cycle while it decreased in ‘Cristobalina’ after dormancy release (Figure 6). Throughout growth cessation and early stages of dormancy, ABA-GE content was significantly higher in the early cultivar ‘Cristobalina’ compared to the late cultivar ‘Regina’ (Figure 8.6).

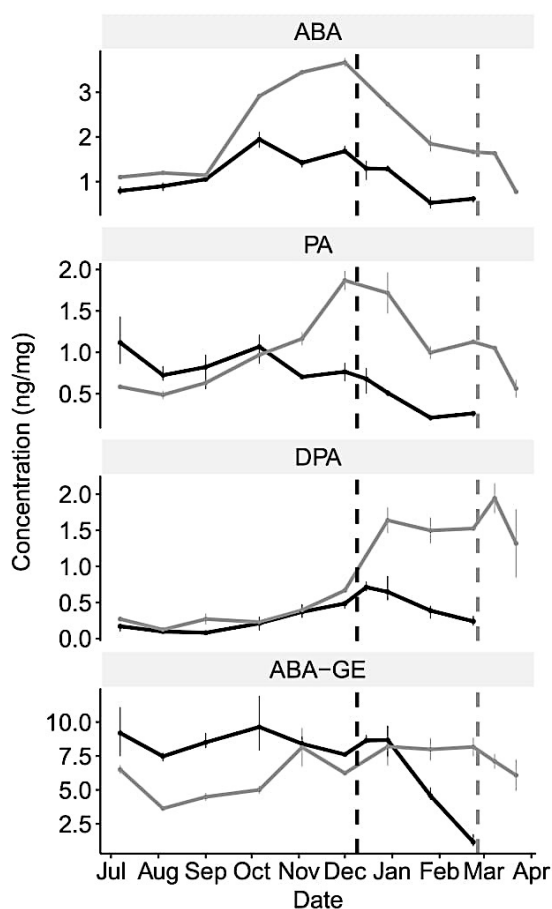


Figure 8.6 | Levels of endogenous bioactive ABA and ABA conjugates in the flower buds of two sweet cherry cultivars during bud development.

Black: ‘Cristobalina’, grey: ‘Regina’. Error bars indicate the data range between the biological replicates (n=3 for ‘Cristobalina’, n=2 for ‘Regina’). Dotted lines represent dormancy release. ABA: Abscisic acid; PA: phaseic acid; DPA: dihydrophaseic acid, ABA-GE: ABA-glucose ester.

8.3.5 Analysis of differentially expressed genes involved in ABA pathway

Expression patterns over flower bud dormancy for genes involved in the multiple ABA biosynthesis steps are shown in [Figure 8.7](#) (*PavNCED1*, 3, 4 and 5) and [Supplementary Figure 8.1](#) (*PavABA1*, 2, 3, and 4). Expression for *PavABA1-4*, *PavNCED1* and *PavNCED4* genes are not correlated with ABA levels while *NCED3* and *PavNCED5* show expression patterns of interest during dormancy. In particular, *PavNCED5* expression is highly correlated with ABA levels, showing a sharp increase during dormancy onset, but also a marked decay before dormancy release. An increase in *PavNCED3* expression occurs when *PavNCED5* expression decreases. On the other hand, ABA 8'-hydroxylases (*CYP707A* genes) are considered the major regulatory enzymes for ABA catabolism, and indeed, low ABA levels are associated with increased expression for *PavCYP707A1* and *PavCYP707A4* before and after dormancy respectively ([Figure 8.7](#), [Supplementary Figure 8.1](#)). By contrast, *PavCYP707A2* is characterised by a sharp increase during endodormancy, more specifically in December, but it is not related to a visible decrease in ABA content. In addition, we identified *PavUGT71B6* ([Supplementary Table 9.1](#)), a sweet cherry orthologous of the *Arabidopsis thaliana* UDP-glycosyltransferase 71B6, that preferentially glycosylates ABA into ABA-GE (Priest *et al.*, 2006). *PavUGT71B6* was considerably upregulated in the early cultivar compared to the late cultivar, with a gradual increase between July and deep dormancy followed by a decrease in expression before endodormancy release ([Figure 8.7](#)). We examined sweet cherry gene predictions for genes involved in ABA signalling. Among the seven ABA receptors PYR/PYL genes identified for sweet cherry ([Supplementary Table 9.1](#)), six were differentially expressed during flower bud dormancy ([Figure 8.7](#), [Supplementary Figure 8.1](#)). In particular, *PavPYL8* expression was correlated with ABA levels, increasing after dormancy onset and decaying after dormancy release ([Figure 8.7](#)). A similar pattern was observed for *PavPYL12* in 'Cristobalina' but not for 'Regina' that exhibited very low expression level. Twelve ABA response genes were identified ([Supplementary Table 1](#)) and seven were differentially expressed ([Figure 8.7](#), [Supplementary Figure 8.1](#)). *PavABF2* and *PavHAB2* had similar patterns with a sharp increase in expression in December while expression of *PavABF3* and *PavABI1* was high from the first sampling points in July to dormancy release when the expression of both genes decreased, one month earlier in 'Cristobalina' than 'Regina'.

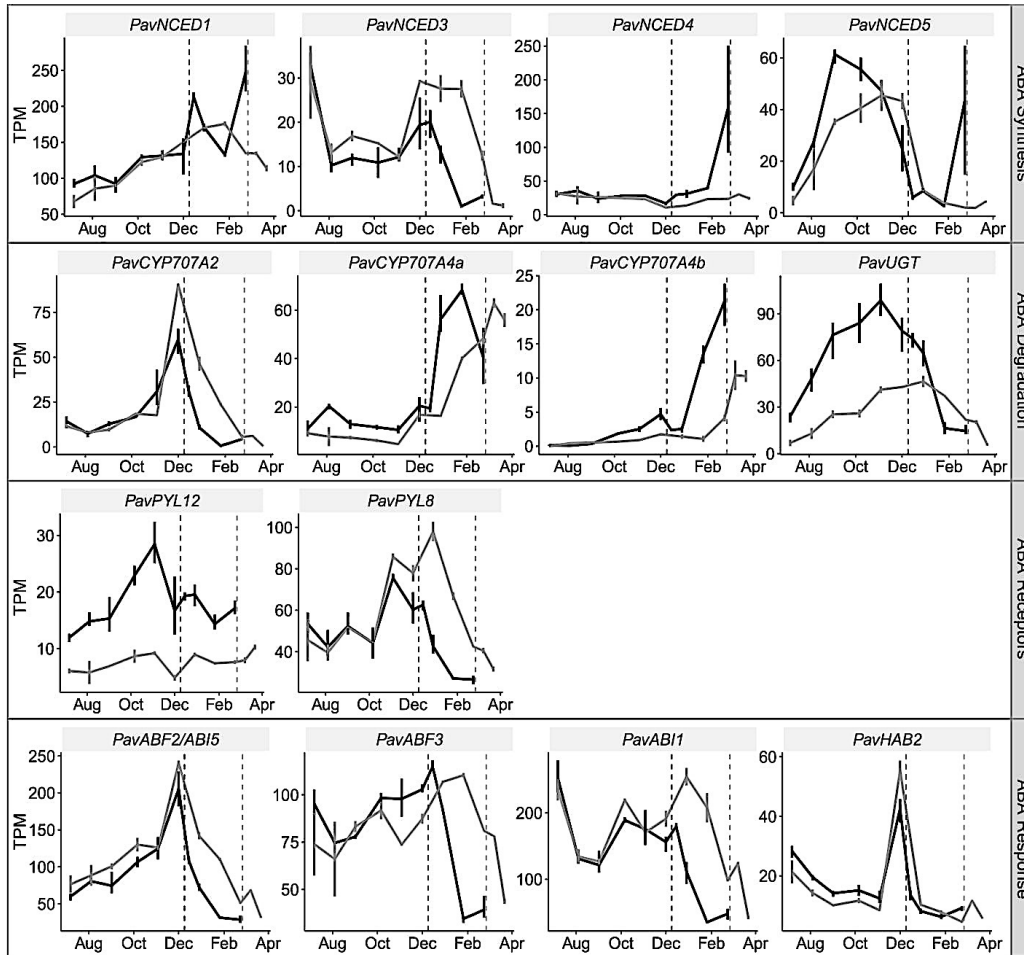


Figure 8.7 | Transcriptional dynamics of genes associated with ABA pathway in the flower buds of two sweet cherry cultivars during bud development.

Expression of specific genes involved in ABA biosynthesis pathway, degradation, signal transduction and response are represented in TPM (Transcripts Per kilobase Million). Black: 'Cristobalina', grey: 'Regina'. Error bars indicate the data range between the biological replicates (n=3 for 'Cristobalina', n=2 for 'Regina'). Dotted lines represent dormancy release.

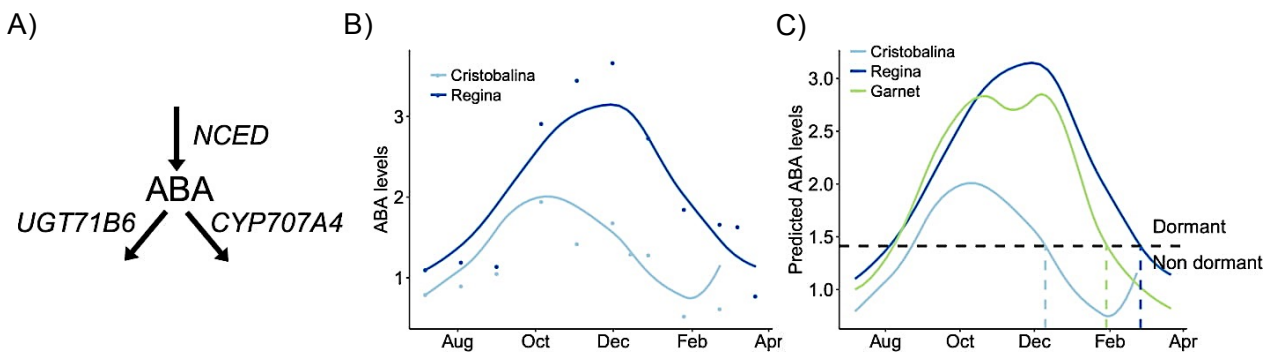


Figure 8.8 | ABA modelling.

(A) Conceptual model used to simulate ABA content. ABA synthesis is controlled by NCED1, NCED3, NCED4 and NCED5 proteins; ABA is deactivated by 8'-hydroxylases CYP707A4 and UGT71B6. The assumption is that enzymatic activity is proportional to gene expression levels. (B) Simulated content of ABA using the model (lines) with means of data (circles) for cultivars 'Cristobalina' and 'Regina'. The model is simulated with parameters: $p_{51,2}=0.009599$, $p_{11,2}=0.0029$, $p_{31,2}=0.002241$, $p_{41,2}=0.0000218$, $p_{51,2}=0.00010859$, $p_{61,2}=0.00012048$ and $p_{71,2}=0.0003027$. (C) Simulated levels of ABA for cultivars 'Cristobalina' and 'Regina' that were used to calibrate the model and 'Garnet'. The model is based on the assumption that ABA levels control the dormancy status: a threshold for ABA levels estimated at 1.412 ng/mg (black dash line) defines whether buds are dormant or non dormant. Simulated dates of dormancy release for 'Cristobalina', 'Garnet' and 'Regina' are December 10th, January 29th and February 26th, respectively (coloured dash lines).

8.3.6 Modelling suggests ABA levels control onset and duration of dormancy

Based on observations that ABA levels are correlated with dormancy depth, ABA content has been proposed as an indicator to assess dormancy status in sweet cherry (Chmielewski *et al.*, 2017). We further investigated the dynamics of ABA to estimate dormancy onset and duration. First, we assumed that ABA synthesis is mainly controlled by *PavNCED1*, 3, 4 and 5 and ABA is converted to PA by 8'-hydroxylases *PavCYP707A4* and esterified into ABA-GE by *PavUGT71B6* (Figure 8.8 A). Since data for protein activity are not available, we used transcript levels as a proxy for enzymatic activity. We then used an Ordinary Differential Equation (ODE) approach to model how dormancy may be regulated by ABA levels. The mathematical model, based on identical parameter set for both cultivars, shows a good fit to the data, indicating that the differential in ABA levels between the two cultivars can be solely explained by the differences in gene expression of the relevant enzymes (Figure 8.8 B). To validate the model, we simulated ABA levels for a third cultivar 'Garnet' that was examined along 'Cristobalina' and 'Regina'. Endodormancy was released on January 21st for 'Garnet', an early flowering cultivar. Based on expression data for *PavNCED1*, 3, 4 and 5, *PavCYP707A4a*, *PavCYP707A4b* and *PavUGT* genes (Supplementary Figure 8.2), the model simulated ABA levels for 'Garnet' during dormancy (Figure 8.8 C). Simulated ABA content for 'Garnet' increases during dormancy onset, reaches high values during endodormancy and decreases before dormancy release, earlier than for the late cultivar 'Regina'. Highest estimated levels for ABA are lower in 'Garnet' than in 'Regina' but higher than 'Cristobalina'.

Results for 'Cristobalina' and 'Regina' cultivars show that levels of endogenous ABA at the date of dormancy release are very similar (Figure 8.6). Consequently, we propose that a threshold for ABA concentration determines the dormancy status and this threshold is constant between cultivars (Figure 8.8 C): ABA levels are low before dormancy onset but as they increase, the ABA threshold triggers dormancy onset; high ABA levels maintain dormancy but they decrease under chilling temperatures and endodormancy is released when ABA content falls below the threshold. The simulated ABA levels for 'Cristobalina' and 'Regina' were 1.414 ng/mg and 1.411 ng/mg at dormancy release, respectively. An average value of 1.412 ng/mg is proposed for the ABA threshold (Figure 8.8 C). Accordingly, simulated dates of dormancy release are December 10th, January 29th and February 26th for 'Cristobalina', 'Garnet' and 'Regina' respectively (Figure 8.8 C). Predicted dates therefore differ from observed dormancy release dates by one, eight and zero day for 'Cristobalina', 'Garnet' and 'Regina', respectively. Since we can account for the dormancy behaviour of a wide-range of cultivars based on the expression of a small number of key genes regulating

ABA levels, this underscores the central role of this phytohormone in the control of dormancy progression.

8.4 Discussion

8.4.1 Sweet cherry specific GA signalling during bud dormancy

We have shown that GA₄ has significant dormancy alleviating effects, similar to previous observations in hybrid aspen (*Populus tremula* × *Populus Tremuloides*; Rinne *et al.*, 2011) and Japanese apricot (*Prunus mume*; Zhuang *et al.*, 2013). Interestingly, results for GA₃ were similar, with a significant increase in bud break under forcing conditions, similar to dormancy alleviating effects observed in peach (Donoho and Walker, 1957) but opposed to null or inhibitory effects on dormancy release previously reported (Rinne *et al.*, 2011; Zheng *et al.*, 2018). In addition, using 20 mg of tissue, high levels of GA₄ and GA₇ were detected in sweet cherry flower buds whereas GA₁ and GA₃ were undetectable in the samples. These observations differ from results obtained on grapevine buds with high GA₁ levels during dormancy induction (Zheng *et al.*, 2018) or on Japanese apricot, characterised with elevated levels of GA₃, especially during ecodormancy (Zhang *et al.*, 2018). Furthermore, we recorded changes for GA₄ levels in sweet cherry flower buds, with a gradual increase during endodormancy and lower values after dormancy release, whereas the lowest GA₁, GA₃ and GA₄ levels were observed during endodormancy in Japanese apricot buds (Zhang *et al.*, 2018). Contradictory observations on GA regulation during dormancy are consistent with the hypothesis that GAs act in a complex manner, with differential effects depending on concentrations and developmental phases, as discussed in Zheng *et al.* (2018). Nonetheless, our results suggest that GA₄ might be the major bioactive GA involved in bud dormancy in sweet cherry.

8.4.2 Three dormancy phases as defined by gibberellin signalling

It has been proposed that GAs have key but distinct functions over bud dormancy progression, especially for the control of dormancy induction and bud break. Here, we define three phases in GA signalling involving different transcriptional cascades, as revealed by our results on GA content and expression of GA pathway-related genes:

i) During bud development and early stages of dormancy (July to October), GA₄ and GA₇ are produced, following an increase in *PavGA20ox2*, along with the upregulation of receptors *PavGID1C* and *PavGID1B* in response to rising GA content as shown in Arabidopsis (Sun, 2011), and the activation of GA signalling, as revealed by the increased expression of GA-response genes *PavGASA4*, *6b*, *6a*, and *9*. High levels of bioactive GA₁ were also detected in grapevine buds during dormancy induction (Zheng *et al.*, 2018). Enhanced GA content triggers the expression of *PavGA2ox8c* and *PavGA2oxb* to catalyse GA deactivation as part of the feedback regulation (Yamaguchi, 2008). In Arabidopsis, proteins GASA4 and GASA6 are involved in flower development and cell elongation, respectively, in response to GA signalling (Roxrud *et al.*, 2007; Zhong *et al.*, 2015) therefore we can hypothesise that gibberellin signalling in this phase controls flower bud development through flower organogenesis.

ii) In November, dormancy depth is at its maximum, characterised by high GA₄ and GA₇ contents and a peak in *PavGA3oxa* expression. Gibberellin homeostasis seems to be maintained during deep dormancy through enhanced expression of deactivation genes *PavGA2ox1* and *PavGA2ox8b*, in 'Regina' and 'Cristobalina' respectively. Interestingly, these contrasted patterns between the two cultivars suggest that GAs may be differentially degraded between cultivars resulting in the accumulation of different types of GA in sweet cherry flower buds. In agreement with acute GA deactivation, only one GA-response gene is activated during this phase, *PavGASA1a*, thus suggesting a very specific response. Interestingly, in grapevine buds, GA₁ content decreased during this phase, defined as dormancy maintenance (Zheng *et al.*, 2018).

iii) After dormancy release, namely ecodormancy phase, levels for GA₄ and GA₇ decrease, associated with low, if not null, expression for *PavGA20ox2* and *PavGA3ox2a* synthesis genes, as well as *PavGA2ox8b*, *PavGA2ox8c*, *PavGA2oxb* deactivation genes and *PavGID1B* and *PavGID1C* receptors. Interestingly, the seven GA-response genes are activated during the ecodormancy, thus suggesting that GA-stimulated pathways are up-regulated. Indeed, another set of genes seems to be activated with enhanced expression for *PavGA20ox1a*, *PavGA2ox1* and genes coding for DELLA proteins.

8.4.3 GA regulation by deactivation during dormancy

Overall, despite increasing GA levels during dormancy, GA-response pathways seem to be mostly activated when *GA2ox* genes are down-regulated after dormancy release so further investigation on the control of GA deactivation during dormancy could unravel a potential

regulation by cold accumulation. One aspect that should be considered about the GA pathway regulation is the critical homeostasis controlled by the DELLA proteins, which are GA signalling repressors that modulate all aspects of GA response. In particular, in Arabidopsis, GA biosynthesis and receptor are among the targets for DELLAs, thus creating a feedback regulation where DELLAs directly impacts GA balance (Zentella *et al.*, 2007). Up-regulation of sweet cherry DELLA-related genes after dormancy release is associated with a decrease in the expression of *PavGID1B*, *PavGID1C* and *GA3ox1*. Therefore, our results suggest that the GA pathway is down-regulated through a feedback loop during dormancy, resulting in a lower GA content in ecodormancy. This hypothesis is also supported by the negative correlation between *PavRGA*, *PavRGA2* and *PavRGL2* expression and GAs content, especially during endodormancy. Similarly, DELLA-annotated genes were also found to be down-regulated during endodormancy and upregulated in dormancy onset and budburst in Chinese cherry (*Prunus pseudocerasus*; Zhu *et al.*, 2015) and Japanese apricot (Zhang *et al.*, 2018). It was shown in Arabidopsis that DELLA proteins are targeted for ubiquitination and degradation by the 26S proteasome, through a direct interaction with the receptor GID1 (Ueguchi-Tanaka *et al.*, 2007) and to the F-box protein SLEEPY1 (SLY1) (Gagne *et al.*, 2002). Consistently, the simultaneous increase in the expression of DELLA-related genes and decrease in *PavGID1B* and *PavGID1C* expression after dormancy release results in the accumulation of DELLA proteins, therefore potentially leading to lower GA levels.

In the model proposed by Zhang and colleagues (2018) for the control of dormancy in Japanese apricot, cold temperatures trigger the expression of *C-repeat BINDING FACTOR (CBF)* genes, therefore promoting cold acclimation through accumulation of DELLA proteins and GA content reduction (Achard *et al.*, 2008). However, our results contradict this model since DELLA-related genes seem to be down-regulated during deep dormancy and activated after dormancy release. In particular, in our system, levels for active GAs are high while buds are dormant so the results question the role of GAs in growth cessation and resumption. In grapevine buds, application of GA₃ inhibits bud break (Zheng *et al.*, 2018) so it is possible that at the detected concentrations, GAs may have a role in dormancy induction and maintenance.

8.4.4 Dormancy depth is correlated with endogenous ABA content

We tested the effect of exogenous application of ABA and fluridone on the sweet cherry bud dormancy status. Buds were not affected by ABA treatment, which could indicate that

endogenous ABA levels in the buds were at the optimal efficiency therefore additional ABA had no effect on dormancy maintenance. Indeed, although delayed bud break was reported after ABA application in birch, sour cherry, grapevine and pear (Mielke and Dennis, 1978; Rinne *et al.*, 1994; Zheng *et al.*, 2015; Li *et al.*, 2018), further assays showed that ABA treatment was effective only during endodormancy since ABA treatment during dormancy release and ecodormancy have no effect on grapevine buds (Zheng *et al.*, 2015). Besides, we observed that dormancy release was triggered in buds treated with fluridone that inhibits ABA biosynthesis and therefore with lower ABA levels, thus suggesting that high ABA levels act to maintain dormancy. This was further confirmed by the contrasted ABA levels over dormancy progression and between low- and high-chill cultivars. Indeed, elevated ABA levels are correlated with deep dormancy in the late cultivar while ABA levels stay low in the early cultivar. We propose that dormancy is triggered and maintained when ABA levels are above a threshold. Bud dormancy may subsequently be released if ABA levels fall below the threshold. Consequently, such a dormancy release threshold would be reached earlier in the season for early cultivars with less ABA (Wen *et al.*, 2016) or when ABA levels are lower due to various environmental conditions (Chmielewski *et al.*, 2017).

ABA levels are also correlated with the expression patterns for genes involved in ABA biosynthesis and degradation. We show that during early stages of dormancy, an increase in *PavNCED5* expression leads to the synthesis of ABA in both cultivars. When *PavNCED5* expression decreases, *PavNCED3* increases, thus maintaining high ABA levels in sweet cherry flower buds. ABA receptors (*PavPYL12*, 8) are highly expressed during deep dormancy as well as ABA response genes. Genes involved in ABA degradation (*CYP707A*) were highly expressed during dormancy release and positively correlated with a decrease of ABA content. Similar results were reported in other *Prunus* species (Wang *et al.*, 2016; Zhang *et al.*, 2015; Tuan *et al.*, 2017) confirming the major role of ABA signalling pathway dormancy. Interestingly, *PavCYP707A2* is up-regulated during deep dormancy for both cultivars, synchronised with the sharp increase in *PavNCED3* expression, thus suggesting a tight regulation of ABA homeostasis in response to low temperatures recorded during this period (Figure 8.3 B). Based on their synchronised peak of expression, it is possible that *PavABF2* and *PavHAB2* are also involved in this acute ABA signalling response to severe cold rather than dormancy progression. Our results thus suggest that two distinct ABA pathways can be distinguished between dormancy regulation and response to cold stress.

8.4.5 A contrasted balance between ABA synthesis and conjugation may explain the differences between early and late cultivars

Overall, although the dynamics of expression for *PavNCED* and *PavCYP707A* genes effectively explain the increasing and decreasing pattern of ABA levels between dormancy induction and release, we further investigated whether they could account for the significant differences observed for ABA levels between the two cultivars. Indeed, *PavNCED3* is more expressed in the late cultivar 'Regina', especially after December, which corresponds to the beginning of ABA decay. However, the difference in *PavNCED3* expression for the two cultivars is less pronounced at the beginning of endodormancy (October, November) when ABA accumulation is noticeably contrasted. Therefore, the higher expression for *PavNCED3* is not sufficient to explain the higher ABA content in 'Regina'. In terms of catabolism, *PavCYP7074a* and *PavCYP7074b* are upregulated earlier in 'Cristobalina', thus accounting for the earlier decrease in ABA levels after dormancy release but not during endodormancy. Differences in ABA catabolites between cultivars were also observed. PA and DPA levels followed the same pattern as ABA but with one-month delay and the ABA catabolites were higher in the late cultivar 'Regina'. Interestingly, Weng and colleagues (2016) exposed the compensatory effect of PA under low ABA conditions, in which PA is recognised by ABA receptors (PYL5 and 2) allowing a supplementary growth inhibition effect. In sweet cherry, increased *PavCYP707A2* expression induces higher level of PA in dormancy in 'Regina' and might therefore result in even deeper dormancy by the inhibitory combination of ABA and PA. Since free ABA levels may be controlled through conjugation as well (El Kayal *et al.*, 2011; Chmielewski *et al.*, 2018), we investigated the conversion of ABA to ABA-GE and we found a *PavUGT71B6* gene characterised by strikingly higher expression levels for the early cultivar compared to the late cultivar. UGT71B6 orthologous in Arabidopsis and Adzuki bean act specifically for ABA conjugation into ABA-GE (Xu, 2002; Priest *et al.*, 2006), so up-regulation of *PavUGT71B6* expression in 'Cristobalina' during dormancy explains the higher content of ABA-GE. We can therefore hypothesise that low ABA content in the early cultivar is due to active catabolism of ABA to ABA-GE.

In simplified terms, we propose that the difference of dormancy release dates between these two cultivars is due to the ABA levels present within the buds. In the early stages of dormancy induction, short days and decreasing temperatures activates ABA biosynthesis while active ABA esterification in early cultivars maintains low ABA levels. Endodormancy period is prolonged in the late cultivar by additional ABA synthesis activated by the enhanced expression of *PavNCED3*. In addition, increasing ABA catabolism, indicated by up-regulation of *PavCYP7072A*, allows the accumulation of PA, which can play a growth-limiting role similar to ABA. Consequently, the potential combination of two inhibitory actors (ABA and PA) deepens dormancy and delays dormancy release in late cultivars.

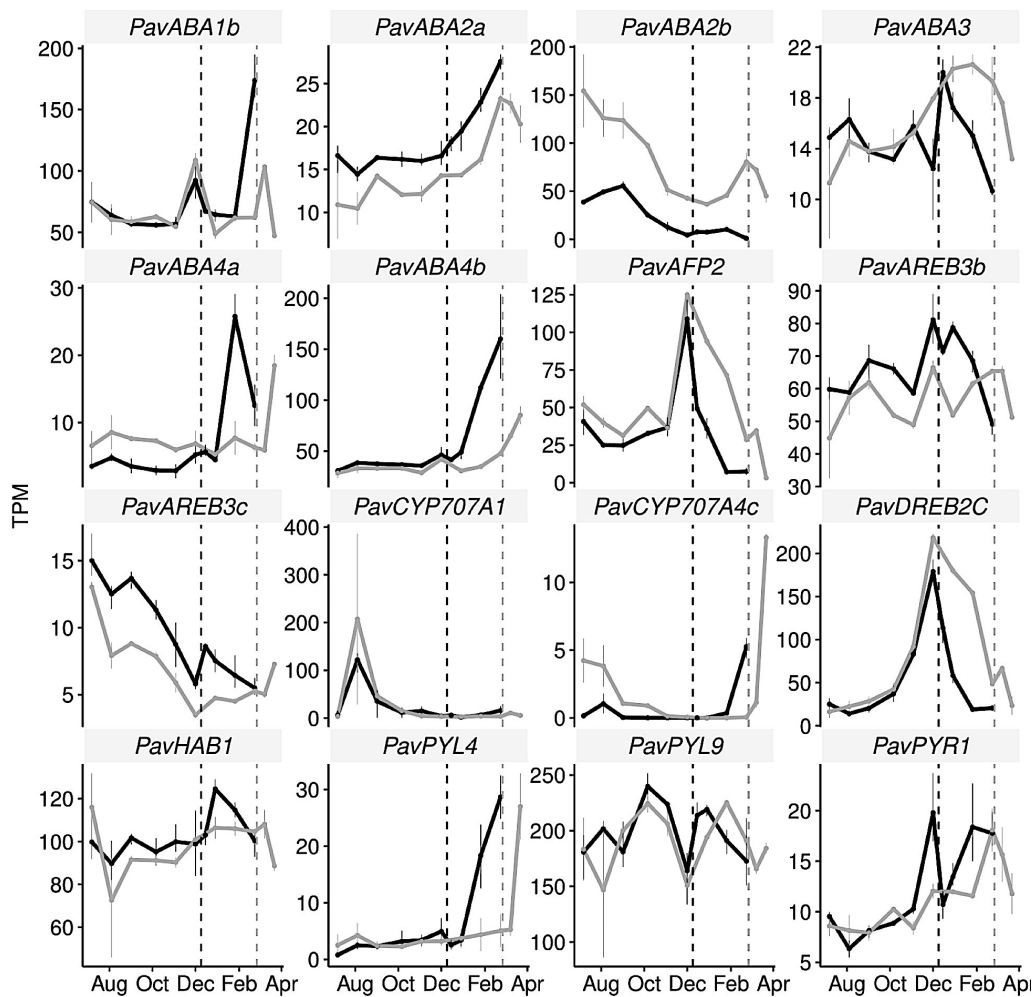
Presently, the remaining question is to elucidate what drives the decrease in ABA levels around dormancy release. Which mechanisms are involved in the down-regulation of *PavNCEDs* and up-regulation of *PavCYP707As* through chill accumulation? Firstly, several reports indicate that ABA might regulate its own accumulation and high levels of ABA attained during endodormancy could up-regulate the expression of catabolic genes such as *PavCYP707A4*, leading to a global decrease in ABA content. Secondly, *DORMANCY-ASSOCIATED MADS-BOX (DAM)* genes have been strong candidates for a key role in dormancy promotion and maintenance since they were identified in the non-dormant *evg* mutant of peach (Rodriguez *et al.*, 1994; Bielenberg *et al.*, 2008). *DAM* genes are highly expressed during dormancy and their expression is inhibited by chill accumulation (Jiménez *et al.*, 2010; Hao *et al.*, 2015), but more interestingly, one example of direct interaction between PpDAM1 and *PpNCED3* promoter was reported in pear (Tuan *et al.*, 2017). Further *in vitro* assays suggest a potential regulation between CBF proteins and *DAM* promoters (Niu *et al.*, 2015; Zhao *et al.*, 2018). We therefore hypothesise that chill accumulation induces *CBF* expression and *CBF* proteins modulate the expression of *DAM* genes that subsequently upregulate *NCED* genes and ABA biosynthesis. Chill accumulation potentially induces chromatin modifications that silences *DAM* genes (Leida *et al.*, 2012b; Ríos *et al.*, 2014; De la Fuente *et al.*, 2015) and inhibits ABA production, consistently with decreasing ABA levels observed as soon as January. Regulation of *DAM* and ABA pathways by cold temperatures should be further investigated to better understand how temperature variations control dormancy progression in sweet cherry flower buds.

8.4.6 Towards new phenology approaches based on molecular mechanisms

Following our observations that ABA levels were highly correlated with dormancy depth and that dynamics of expression for ABA synthesis and catabolism could explain the differences observed between cultivars, we have successfully modelled ABA content and dormancy behaviour in three cultivars exhibiting contrasted dormancy release dates. Indeed, ABA had been proposed as an indicator for dormancy release in sweet cherry (Chmielewski *et al.*, 2017) but to our knowledge, this is the first attempt to simulate dormancy onset and duration using molecular data. Only a small number of key genes regulating ABA were sufficient to account for all variations in ABA levels and dormancy progression overtime and between cultivars. However, previous analyses have shown that ABA levels are highly variables between years (Chmielewski *et al.*, 2017) therefore further analyses are needed to explore and validate the current model.

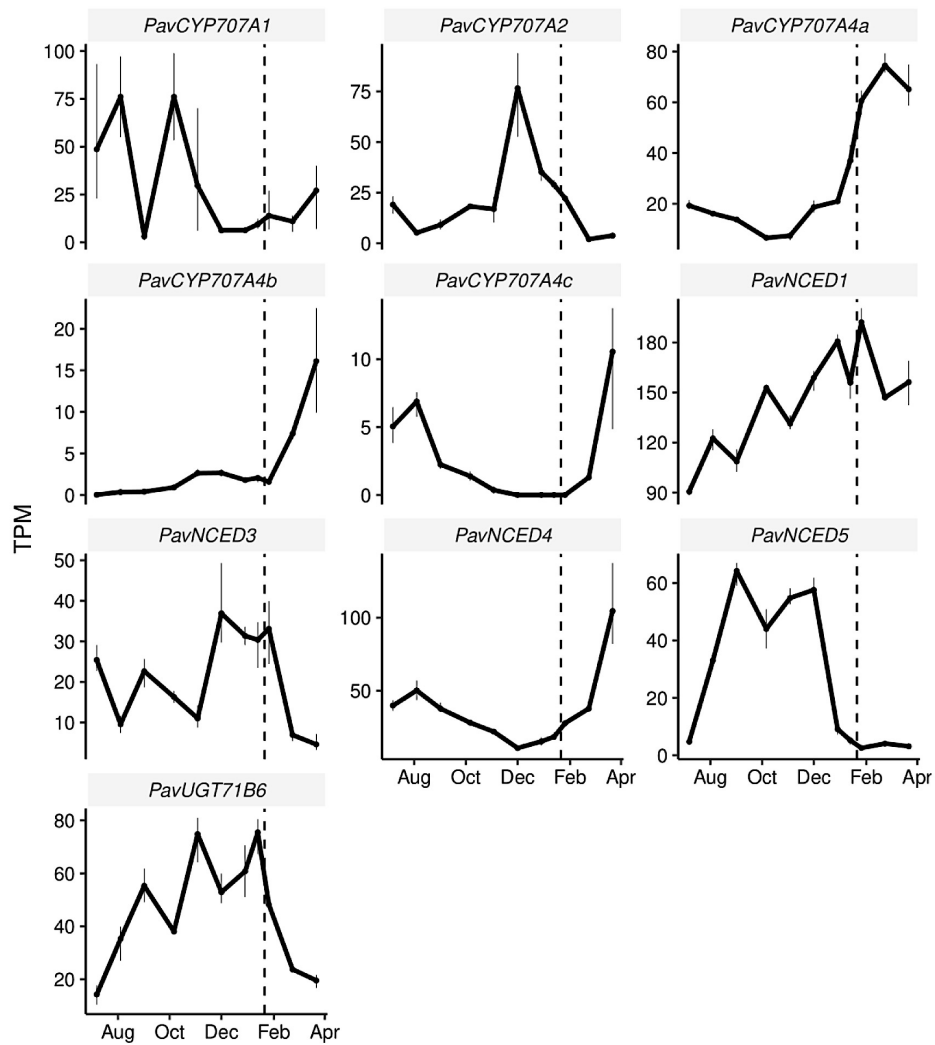
Antagonistic actions of ABA and GA have been extensively studied in seed dormancy (Shu *et al.*, 2018) and the ABA/GA ratio is often proposed as a determinant factor in the control of rest and growth responses, including dormancy release (Zhang *et al.*, 2018). Therefore, integrating GA signalling into the bud dormancy model might be necessary to better account for the regulation of dormancy release. For example, it is possible that high GA levels around dormancy release play a role by overcoming the ABA-dependent growth inhibition. Interaction between GA and ABA pathways might also be critical in the response to environmental conditions during dormancy, including intertwined regulations of hormone biosynthesis (Shu *et al.*, 2013; Yue *et al.*, 2017). This was applied in a very innovative model for seed germination based on the endogenous hormone integration system (Topham *et al.*, 2017). The hormonal balance between GA and ABA is regulated by endogenous and environmental signals towards the developmental switch that triggers termination of dormancy and germination. Accordingly, ON/OFF systems, like dormancy or flower initiation, can be modelled as developmental switches triggered in response to quantitative inputs after a threshold has been reached (Wilczek *et al.*, 2009; Donohue *et al.*, 2015; Bassel, 2016). In our current model, we propose a first step for mechanistic modelling of dormancy onset and release based on expression data and ABA quantification. The next steps, in addition to the integration of GA signalling and its crosstalk with ABA, will be to provide information on temperature-mediated control of the regulatory cascades. Ground-breaking research led on *Arabidopsis*, allowed by high-throughput sequencing techniques, has hastened the pace for the incorporation of molecular data into phenology models (Satake *et al.*, 2013; Kudoh, 2016), thus opening new roads for perennial studies (Chuine and Régnière, 2017).

Supplemental figures



Supplementary Figure 8.1 | Transcriptional dynamics of genes associated with ABA pathway in the flower buds of two sweet cherry cultivars during bud development.

Expression of specific genes involved in ABA biosynthesis pathway, degradation, signal transduction and response are represented in TPM (Transcripts Per kilobase Million). Black: 'Cristobalina', grey: 'Regina'. Error bars indicate the data range between the biological replicates (n=3 for 'Cristobalina', n=2 for 'Regina'). Dotted lines represent dormancy release.



Supplementary Figure 8.2 | Transcriptional dynamics of genes involved in ABA synthesis and degradation for the sweet cherry cultivar 'Garnet'.

Expression are represented in TPM (Transcripts Per kilobase Million). Black: 'Cristobalina', grey: 'Regina'. Error bars indicate the data range between the biological replicates (n=3). Dotted lines represent dormancy release.

To protect the endangered cherry production due to climate change, one strategy is the application of biostimulants to counteract insufficient chilling requirements arising as a result of mild winters. We tested several biostimulants, from the Roullier Group, on sweet cherry dormant buds to see their impact on dormancy release. The results for one of the best candidates are exposed in the following chapter. This publication is the basis for the creation of a patent with the Roullier Group.

9. OKEN: a new product impacting bud break in sweet cherry (*Prunus avium* L.)

Noémie Vimont^{1,2,3}, Adrian Schwarzenberg², Mustapha Arkoun², Frank Jamois², Jean-Claude Yvin², Philip A. Wigge³, Elisabeth Dirlwanger¹, Bénédicte Wenden¹

¹UMR 1332 BFP, INRA, Université de Bordeaux, F-33140 Villenave d'Ornon, France

²Agro Innovation International - Centre Mondial d'Innovation - Groupe Roullier, 35400 St Malo, France

³The Sainsbury Laboratory, University of Cambridge, CB2 1LR, United Kingdom

9.1 Introduction

Seasonal timing of phenological events is crucial not only for plant survival but also for maintaining high production in fruit trees. According to some models, as global temperatures rise, harvests in some areas are expected to fall by as much as 6 to 10% for every 1°C increase in temperature (Wenden *et al.*, 2017). In some regions where chilling temperatures are insufficient to overcome endodormancy, incomplete dormancy release has been observed leading to bud break delay and low bud break rate (Erez, 2000) and therefore to low yields (Campoy *et al.*, 2011; Luedeling, 2012). As a consequence, the response to temperature should represent a priority target for breeding strategies. It is particularly relevant for cherry because its dormancy is mainly controlled by temperature (Heide *et al.*, 2008). However, the breeding of new cultivar is a long process lasting up to twenty years and the effect of climate change is already perceived and has dramatically affected sweet cherry production in the south-west of France (G. Charlot, Balandran, France, 2016, personal communication). Unlike other *Prunus* species like peach (*Prunus persica*), no low-chill cherry cultivar is currently available to adapt production to future climatic conditions. One strategy to quickly alleviate the negative impact of climate change on cherry production is the application of dormancy-breaking products in spring to counteract insufficient chilling temperatures to overcome dormancy, which are lacking in autumn-winter, and to decrease disorders of

phenology.

Hydrogen cyanamide (HC), mainly sold as Dormex® (520 g/l hydrogen cyanamide, AlzChem, Trostberg, Germany) is the most successful chemical used to advance and synchronise bud break and is largely used in the scientific community to understand the bud break process in perennials (Or *et al.*, 2000; Abu-Qaoud, 2004; Pérez *et al.*, 2008; Ophir *et al.*, 2009; Yamane *et al.*, 2011; Vergara *et al.*, 2012; Ionescu *et al.*, 2016, 2017). It was reported that HC affects the expression of flowering-related genes, such as *PHYTOCHROME A (PHYA)* and *B (PHYB)*, *FLOWERING LOCUS T (FT)*, *CONSTANS (CO)* (Pérez *et al.*, 2011), *GA STIMULATED ARABIDOPSIS (GASA)* and gibberellin-related genes (Zheng *et al.*, 2018). HC seems to increase the bud oxidative status by increasing the ratio of reduced (GSH) to oxidised (GSSG) glutathione (Pérez *et al.*, 2009) and reducing the catalase activity (Ionescu *et al.*, 2017), which are two key components for the detoxification of hydrogen peroxide. It further increased the level of proline (Mohamed *et al.*, 2011) and ethylene (Ophir *et al.*, 2009, Ionescu *et al.*, 2017) and decreased ABA levels (Zheng *et al.*, 2015) and indole-3-acetic acid (IAA) levels in buds (Ionescu *et al.*, 2017). Nonetheless, Dormex® has been banned in France and other European countries as it was demonstrated that Dormex® has a negative impact on human and animal health (Schep *et al.*, 2008; Sheshadri *et al.* 2011). For this reason, the Roullier Group aims at finding alternatives to this chemical in collaboration with the National Institute of Agronomic Research (INRA) Bordeaux.

Prior to this study, a screen of eight biostimulants has been tested on cut branches in controlled conditions during two years, as well as in *Arabidopsis thaliana*. The effects of two interesting candidates were confirmed in field, however only OKEN showed the most significant results (Noémie Vimont, Roullier Group, unpublished data). In this chapter, we present the impact of OKEN on sweet cherry dormancy release by different approaches such as phenological observations in controlled and natural conditions, and transcriptomic and metabolomic analyses.

9.2 Material and Methods

Bud break percentage and dormancy status were evaluated using forcing experiments as described in “Materials and methods” section page 52. Products obtained from the Roullier Group are detailed in the “Materials and methods” section page 53 and their effects on dormancy and flowering were tested following application on cut branches under controlled conditions (“Materials and methods” section page 54) or on trees in natural conditions (orchards, “Materials and methods” section page 56). Buds from treated trees were sampled and tissues were used to explore gene expression by qRT-PCR reactions

(“Materials and methods” section page 61), and to quantify phytohormones (“Materials and methods” section page 78) and sulphur metabolites (“Materials and methods” section page 79).

9.3 Results

9.3.1 OKEN stimulates bud break and presents no toxicity for buds

To test the effect of exogenous application of a biostimulant (OKEN) on dormancy status, we sprayed dormant branches of the sweet cherry cultivar ‘Fertard’ with solutions containing surfactant (control), surfactant + OKEN and surfactant + Dormex®. Dormancy status was evaluated by the bud break response to forcing conditions (Figure 9.1). Firstly, it is important to notice that the percentage of bud break, representing the bud dormancy status, differ between years (Figure 9.1; Figure 9.1 B, D). These differences are due to differences in dormancy status for the branches when they were sampled for the assay. Indeed, application assays were conducted at three quarters of chilling requirements but available chilling models may be inaccurate or unable to guarantee comparable chilling status between years for the cultivar ‘Fertard’. Dormex® showed higher percentage of bud break than surfactant in 2015 and 2016 (Figure 9.1 A, B), however for the 2017 and 2018 treatments, Dormex® application seemed toxic to the buds and displayed very low percentage of bud break in 2017 with the presence of bud necrosis (Figure 9.1 C) and all buds were necrotic in 2018 (Figure 9.1 D). For each experiment, OKEN displayed higher percentage of bud break at 0.275%, 0.550%, and this when doing one or two applications, than Dormex® (Figure 9.1 A-D). Nonetheless, OKEN at 1.1% appears to be toxic as the percentage of bud break is always lower than surfactant (Figure 9.1 A). In 2018, different formulations of OKEN were tested (OKEN batch A, B, C and D; Figure 9.1 D). No considerable differences were found between the formulations (Figure 9.1 D). However, OKEN products displayed equivalent percentage of bud break in 2018 than surfactant (Figure 9.1 D) but a strong decrease in 2017 with two applications at 0.550% (Figure 9.1 C).

9.3.2 Two applications of OKEN on sweet cherry trees increases bud break

In order to confirm the effect of OKEN found in controlled conditions, OKEN applications were tested on trees in the field in 2017 and further confirmed in 2018. The experimental design allowed us to compare the effect of one application (1.1%) and two applications (at 0.550%) with one-week interval. Dormancy status and bud break were estimated by forcing branches sampled from the treated trees at regular intervals.

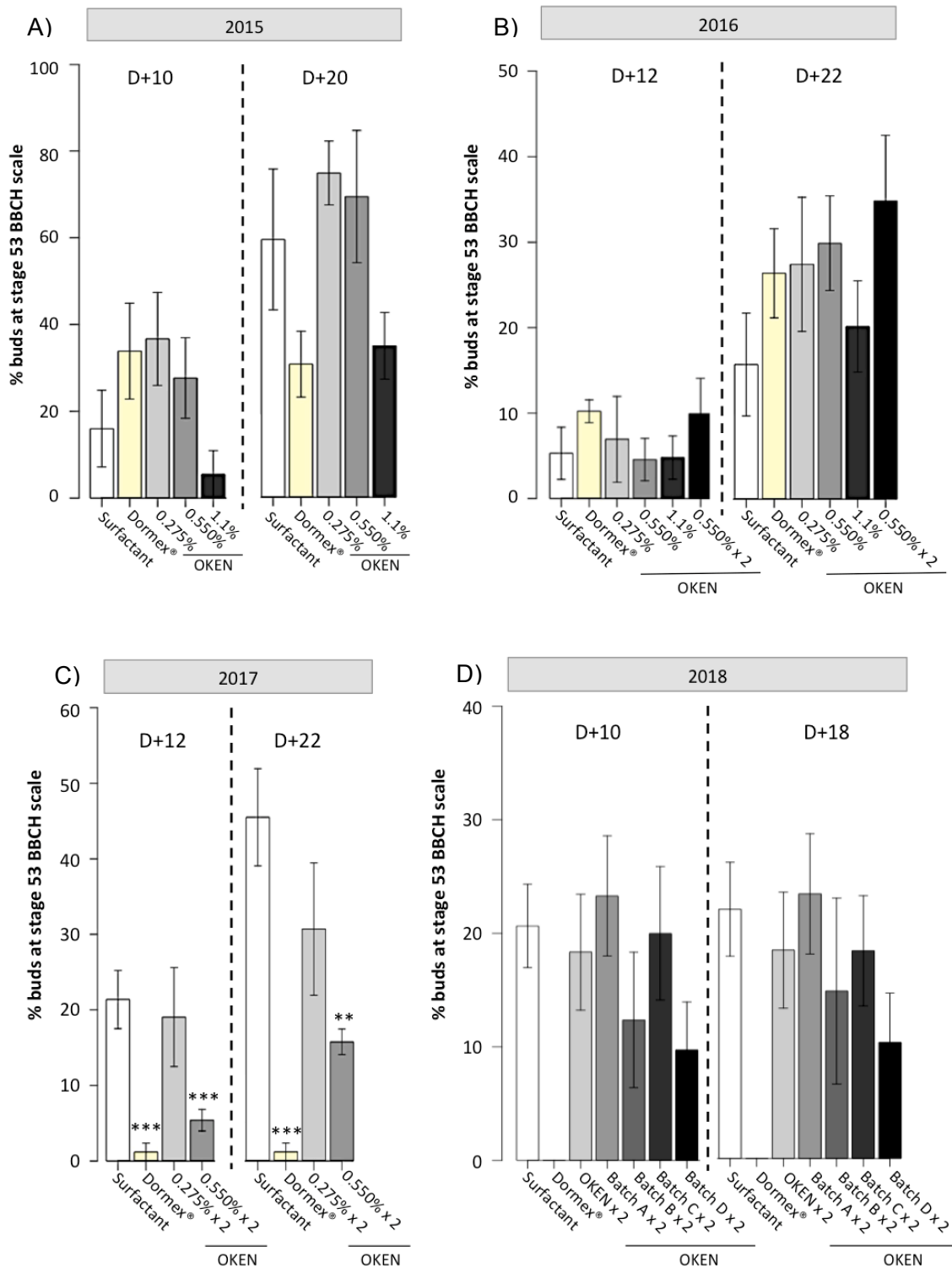


Figure 9.1 | Effect of OKEN on the sweet cherry dormancy status during four years of experiment

Sweet cherry branches ('Fertard' cultivar) were treated with a biostimulant (OKEN) at different concentrations, with one or two applications (x2), different formulations of OKEN (batch A, B, C and D), a surfactant (control) and a dormancy-breaking reagent (Dormex®) and transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded after around 10 days and around 20 days. Error bars indicate the data range between the five biological replicates (n=5). Asterisks indicate treatments that differ significantly from treated branches with a surfactant (Student *t*-test, $p < 0.05$ (*)) and $p < 0.01$ (**)).

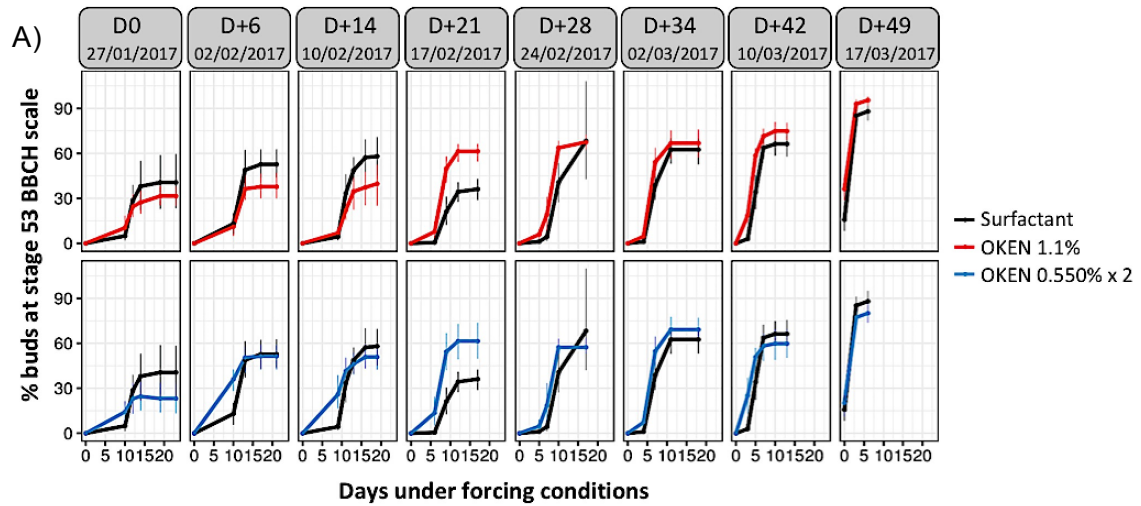


Figure 9.2 | Effect of OKEN on the sweet cherry dormancy status in 2017 in field

(A) Sweet cherry trees ('Fertard' cultivar) were treated with a biostimulant (OKEN) at different concentrations (1.1% and 0.550%), with one or two applications (x2) and a surfactant (control). A harvest time course was carried out to observe the effect of OKEN on dormancy. Branches were transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded over time. Error bars indicate the data range between the six biological replicates (n=6). "D+6" represents the number of days after treatment. (B) Picture of one branch from a tree treated with two applications (x2) of a biostimulant (OKEN) at a concentration of 0.550% on the left and one branch from a tree treated with a surfactant on the right after six days in forcing conditions and harvested the 17/02/2017.

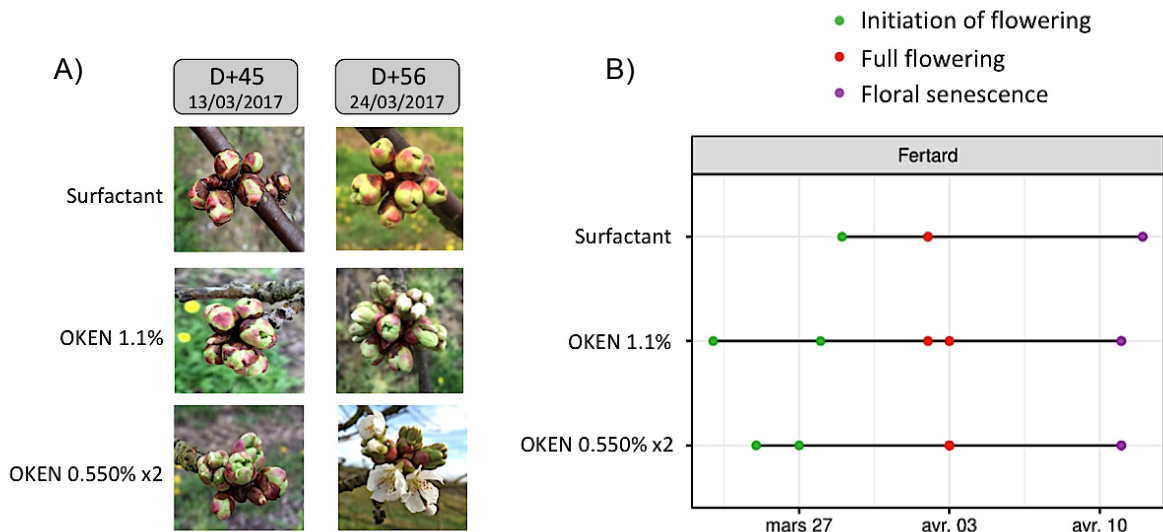


Figure 9.3 | Effect of OKEN on budbreak and flowering time for a late cultivar 'Fertard' in field (2017)

(A) Pictures representing the floral buds status 45 days and 56 days after treatments in field. (B) Effect of different treatments on the date of flowering of floral buds. Circles represent the number of trees at different stages of flowering (n=2 trees).

In 2017, both treatments led to a substantial increase in bud break after 21 days of treatment (Figure 9.2 A). Interestingly, two applications of OKEN at 0.550% rapidly produced higher percentages of bud break than the control when observed 6, 14 and 21 days after treatment (Figure 9.2 A,B). No considerable differences were observed between treatments after 17/02/2016 (D+21). Indeed, as cold temperatures during winter were not lacking, chill requirements were probably satisfied and endodormancy was released for all trees, noticeable by bud break percentage of more than 50%. Nonetheless, observations on the field showed earlier bud break for OKEN treatments compared to surfactant (Figure 9.3 A). These observations were further confirmed by the earlier flowering dates for trees treated with the two OKEN modalities than for the control (Figure 9.3 B). However, no differences between treatments were observed for full and end of flowering (Figure 9.3 B).

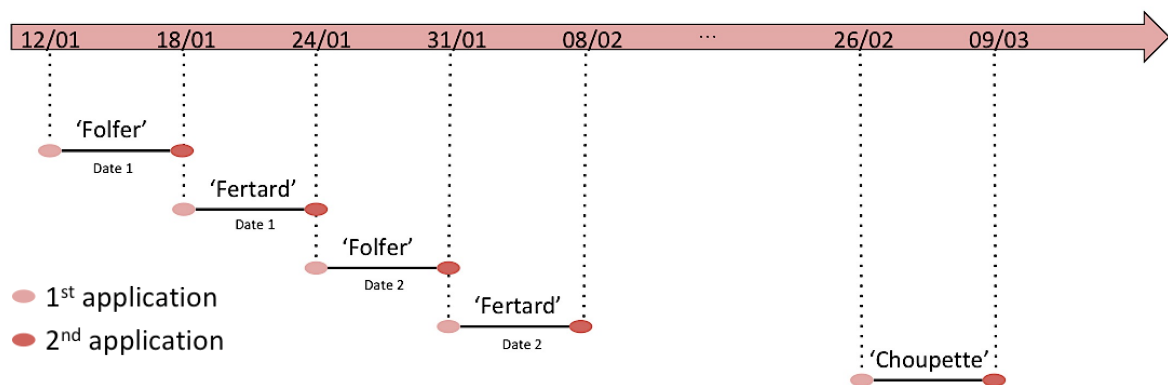


Figure 9.4 | Schedule of treatments for field experiment in 2018

OKEN, different formulations of OKEN (batch A and B) and a surfactant were sprayed on 'Folfer' (early cultivar) and 'Fertard' (late cultivar) trees, when trees had reached three quarter of chilling requirements (Date 1). In addition, OKEN only was sprayed on other 'Folfer' and 'Fertard' trees at a latter date mentioned as Date 2. OKEN and a surfactant were sprayed on 'Chouquette' trees, an apple cultivar. OKEN products were applied with two applications (one week interval) at 0.550% .

To further confirm the effect of OKEN treatments on bud break and dormancy status, different formulations of OKEN (OKEN batch A and B) were tested with the two application modality at 0.550% on two different sweet cherry cultivars and an apple cultivar in 2018 (Figure 9.4). All formulations of OKEN showed similar results (Figure 9.5 A) for the early cultivar 'Folfer'. An increase in the bud break is found for OKEN products compared to surfactant 27 days after treatments when OKEN is applied on trees when they reached three quarter of chilling requirement (Figure 9. 5; Figure 9. A), and after 15 days when OKEN is applied at a later date (Figure 9.5 B). Interestingly, the percentage of bud break decreased over time following a surfactant treatment (Figure 9.5 A, B). This decrease may be due to high bud necrosis caused by a freezing spell in February 2018. However, with OKEN

treatments the percentage of bud break continued to rise (Figure 9.5 A, B). For the late cultivar ‘Fertard’, no differences or opposite effects along sampling dates were found between OKEN treatments and surfactant (Figure 9.6 A, B). Nonetheless, the last harvest (4 April 2018) showed higher bud break percentage when OKEN is applied at a later date (Figure 9.6 B). The flowering date estimation in the field, demonstrated that there was no observable differences between treatments and control (Figure 9.7 A, B). To evaluate the action spectrum of OKEN, this product was tested on an apple cultivar ‘Choupette’ in the field. However, no differences of flowering date were observed between treatments and the control (Figure 9.7 C).

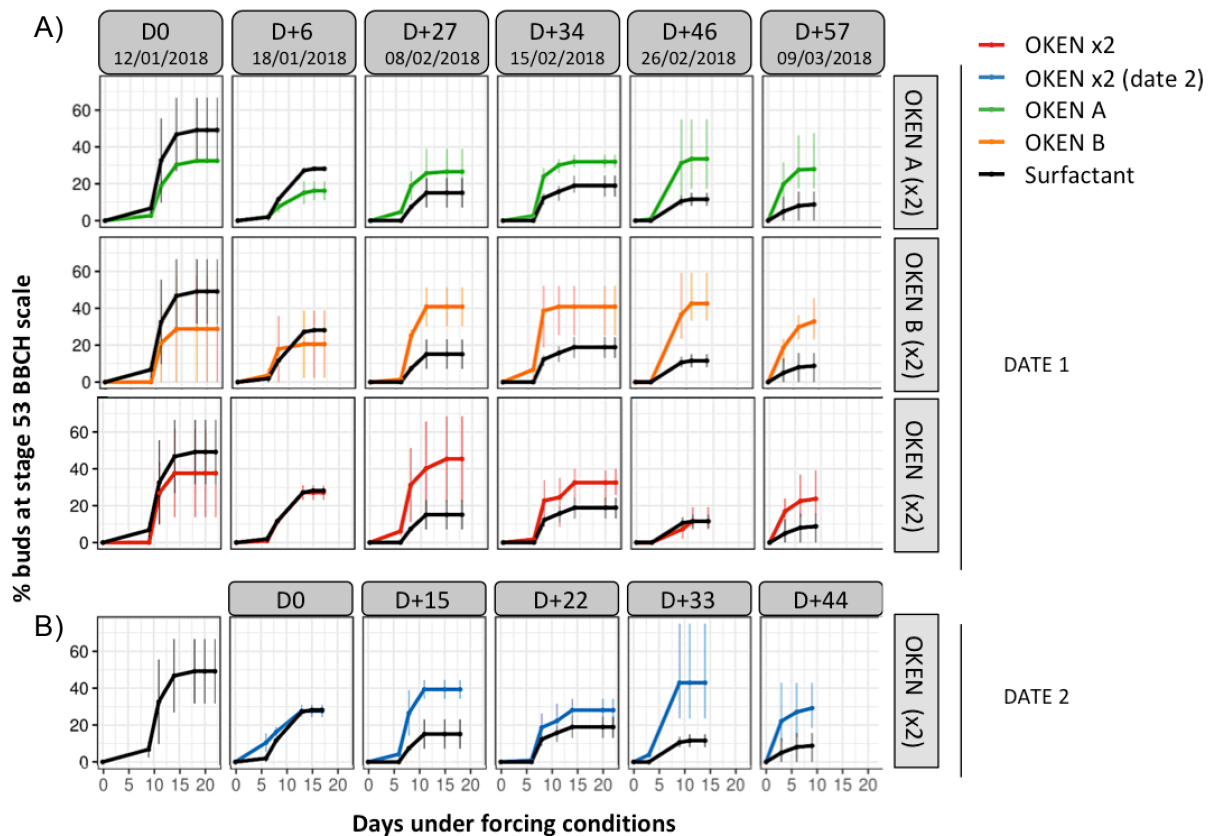


Figure 9.5 | Effect of OKEN on the sweet cherry dormancy status of an early cultivar ‘Folfer’ (2018)
 (A) Sweet cherry trees (‘Folfer’ cultivar) were treated with two applications (x2) of a biostimulant (OKEN) at a concentration of 0.550%, and a surfactant (control) when trees had reached three quarter of chilling requirements (Date 1). (B) And at latter date mentioned as Date 2. A harvest time course was carried out to observe the effect of OKEN on dormancy. Branches were transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded over time. Error bars indicate the data range between the three biological replicates (n=3). “D+6” represents the number of days after treatment.

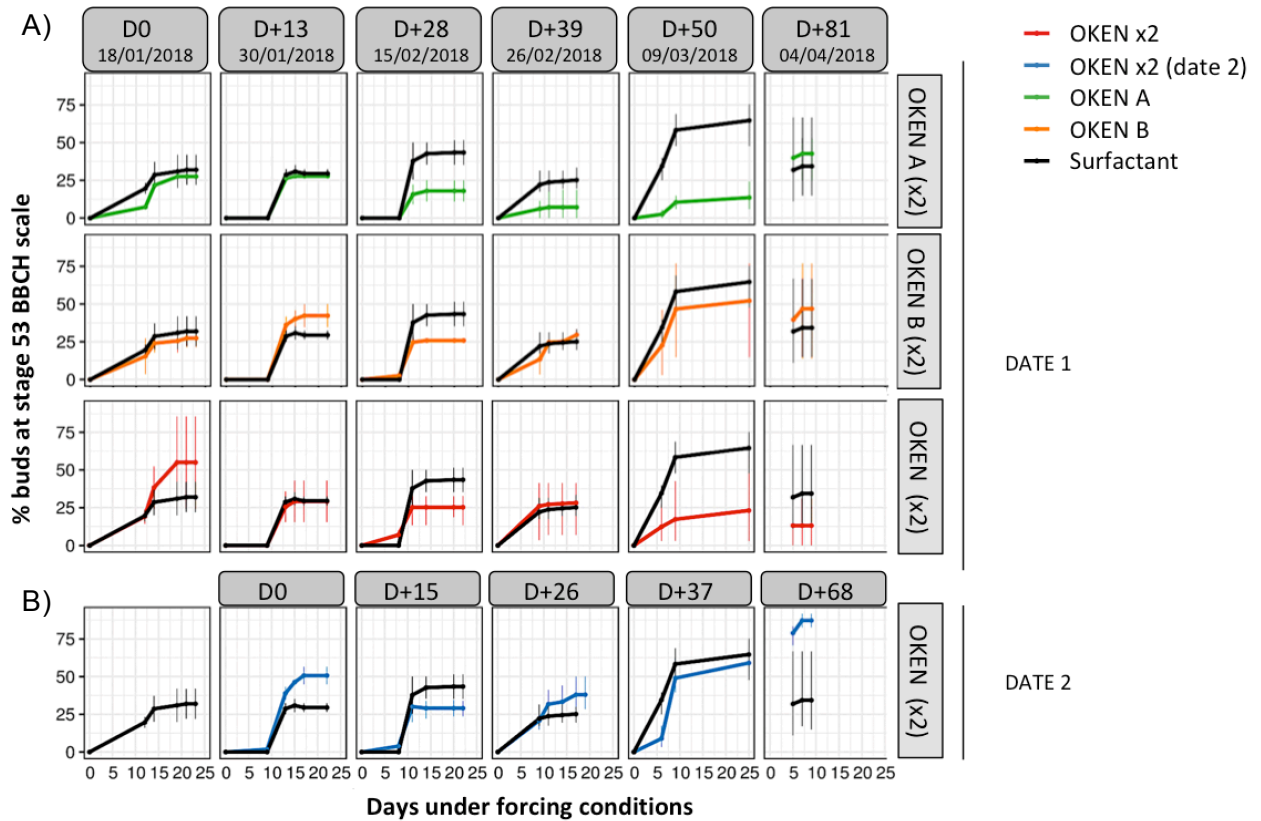


Figure 9.6 | Effect of OKEN on the sweet cherry dormancy status of a late cultivar 'Fertard' (2018)
 (A) Sweet cherry trees ('Fertard' cultivar) were treated with two applications (x2) of a biostimulant (OKEN) at a concentration of 0.550%, and a surfactant (control) when trees had reached three quarter of chilling requirements (Date 1). (B) And at latter date mentioned as Date 2. A harvest time course was carried out to observe the effect of OKEN on dormancy. Branches were transferred under forcing conditions (25°C, 60-70% humidity, 16 hours light). The percentage of flower bud break was recorded over time. Error bars indicate the data range between the three biological replicates (n=3). "D+13" represents the number of days after treatment.

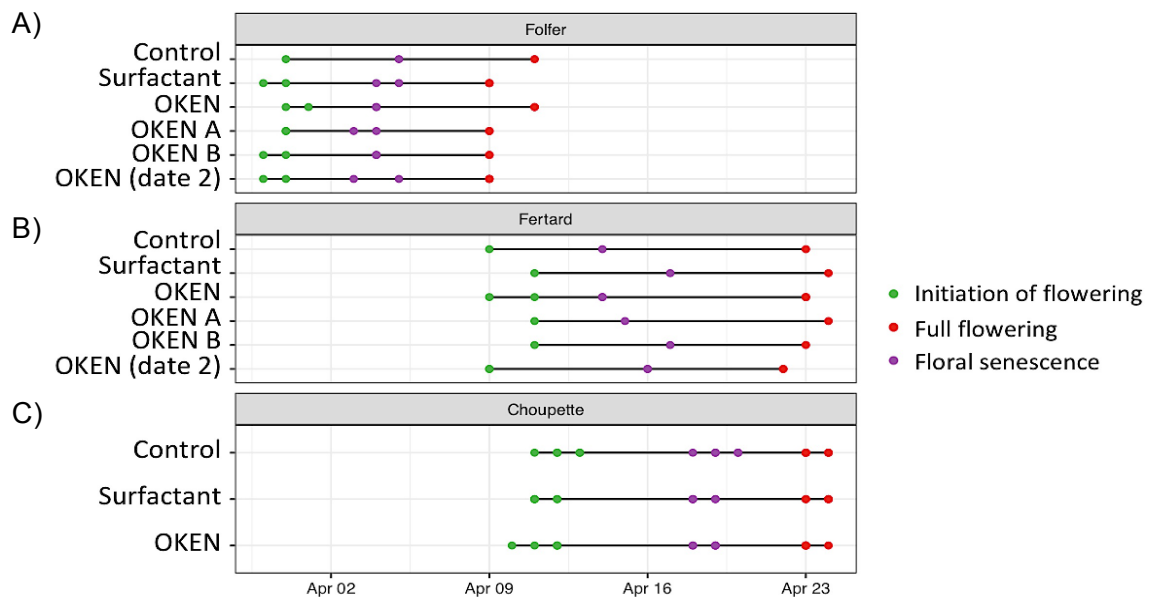


Figure 9.7 | Effect of OKEN and different formulation of OKEN on budbreak and flowering time on a late and an early sweet cherry cultivar and an apple cultivar in field (2018)
 (A) Effect of different treatments on the date of flowering of floral buds of an early sweet cherry cultivars 'Folfer', (B) on a late sweet cherry cultivar 'Fertard' and (C) of an apple cultivar 'Choupette'. Circles represent the number of trees at different stages of flowering (n=2 'Folfer' trees, n=1 or 2 'Fertard' trees and n=3 'Choupette' trees).

9.3.3 OKEN impact the expression of dormancy-related genes and bud metabolism

Transcriptomic and metabolomic analyses were carried out on flower buds from the 2017 field experiment in ‘Fertard’ cultivar.

Two key genes involved in dormancy release and flowering were, *DORMANCY ASSOCIATED MADS-BOX 4* (*PavDAM4*) and *FLOWERING LOCUS T* (*PavFT*), respectively, were tested by qRT-PCR (Figure 9.8). A considerable decrease of *DAM4* expression was observed 14 days after treatment for one and two applications of OKEN compared to surfactant (Figure 9.8 A). The decrease in expression is lower with two applications than with only one (Figure 9.8 A). Conversely, expression for *PavFT* increased 14 days after treatment when buds had been treated with two OKEN applications (Figure 9.8 B). An increase of *FT* expression was also observed for one application but with a time delay (21 days after treatment) (Figure 9.8 B).

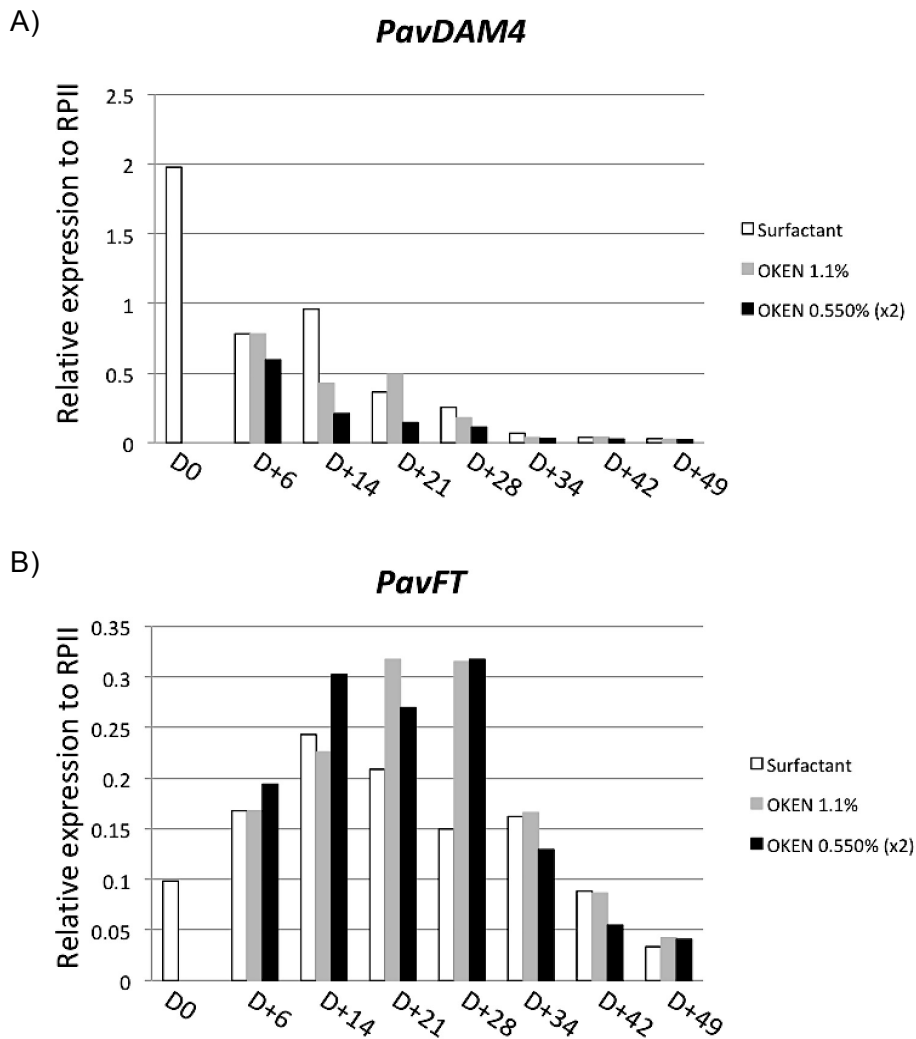


Figure 9.8 | Transcriptional dynamics of genes associated with flowering and dormancy pathway in the flower buds of sweet cherry after OKEN treatments

(A) Expression of *DORMANCY ASSOCIATED MADS BOX 4* (*PavDAM4*) and (B) *FLOWERING LOCUS T* (*PavFT*) were quantified by quantitative real-time PCR and normalised to *RNA POLYMERASE II* (*RPII*) (for one biological replicate).

As shown in chapter 8, hormones have a key role in the control of dormancy progression. Different hormone quantifications, including abscisic acid (ABA), gibberellic acid (GA7 and GA9) and jasmonic acid (JA), were therefore performed buds sampled from trees after the treatments (Figure 9.9). ABA and GA9 levels decreased compared to surfactant 13 days after two applications of OKEN at 0.550% (Figure 9.9 A, C). However, no difference in ABA levels was observed for one application of OKEN compared to surfactant (Figure 9.9 C). A marked effect for both OKEN treatments was observed with a decrease in JA levels 21 days after treatment (Figure 9.9 D). In contrast, OKEN treatments increased levels of GA7 from 6 days after treatment (Figure 9.9 B). We also see that GA7 and GA9 levels increased after 13 days of one OKEN application at 0.550% (Figure 9.9 A, B).

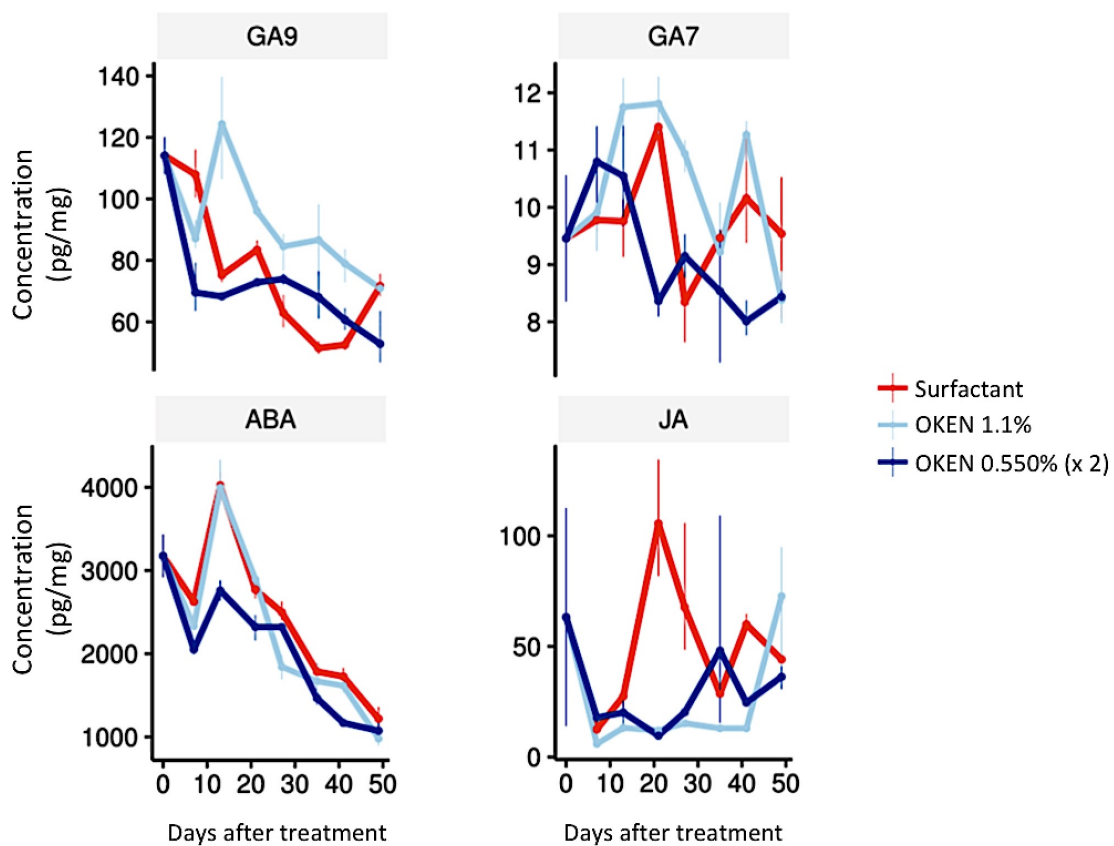


Figure 9.9 | Levels of endogenous phytohormones in the flower buds of sweet cherry after OKEN treatments

Error bars indicate the data range between the three biological replicates (n=3). GA=gibberellic acid; ABA=abscisic acid and JA= jasmonic acid.

Another key signalling pathway during dormancy is mediated by redox status so we evaluated the ratio of reduced to oxidised glutathione (GSH/GSSG), which is a marker of oxidative stress (Figure 9.10). A massive and significant increase of the GSH/GSSG ratio was observed 14 days after two applications of OKEN until 49 days after treatment

compared to the control (Figure 9.10), while one application modality displayed a slightly lower ratio than surfactant (Figure 9.10).

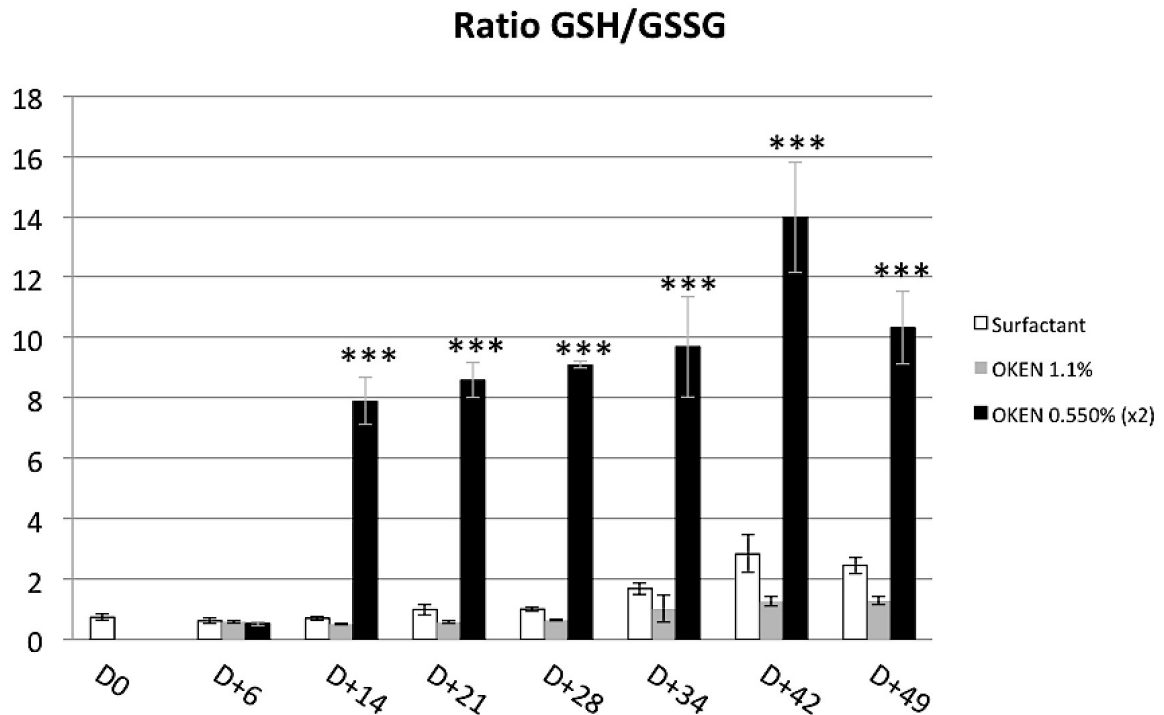


Figure 9.10 | Ratio of reduced to oxidised glutathione (GSH/GSSG) in the flower buds of sweet cherry after OKEN treatments

Error bars indicate the data range between the three biological replicates (n=3). Asterisks indicate treatments that differ significantly from treated branches with a surfactant (Student test, $p < 0.05$). “D+16” represents the number of days after treatment. GSH= reduced glutathione; GSSG=oxidised glutathione

9.4 Discussion

9.4.1 Similar physiological responses between dormancy-breaking reagents Dormex® and OKEN

Hydrogen cyanamide (HC) found in Dormex® is used by the scientific community to understand the process of bud break in several species. Expression of one major gene involved in flowering, *FLOWERING LOCUS T (FT)*, was increased following a HC treatment on grapevine buds (Pérez *et al.*, 2011) as we found after the OKEN treatment (Figure 9.8 B). Conversely, *DAM* genes involved in bud dormancy were down-regulated after HC treatment on peach buds (Yamane *et al.*, 2011) and Japanese pear (Saito *et al.*, 2013). In sweet cherry, *PavDAM4* expression, associated with endodormancy release and bud break (Leida *et al.*, 2010; Sasaki *et al.*, 2011), decreased after an OKEN treatment (Figure 9.8 A). In

addition to these expression changes 13 days after two applications of OKEN, a considerable increase in bud break percentage was observed on the following week (21 days after treatment; [Figure 9.2 A](#)). Taken together, these results suggest that OKEN can overcome dormancy in a Dormex®-like manner.

Pérez *et al.* (2009) demonstrated that glutathione is predominantly in its the oxidized form (GSSG) during endodormancy in grapevine buds and the GSH/GSSG ratio was reversed following HC treatment. Similar results were found following a OKEN treatment ([Figure 9.10](#)), thus suggesting that OKEN and Dormex® overcome endodormancy by the induction of oxidative stress. This hypothesis was also confirmed by an increase in hydrogen peroxide levels following HC treatment (Pérez *et al.*, 2008). A decrease in ABA levels were found in grapevine buds after a HC treatment and are associated with a decrease in expression of *VvNCED1*, a homologue of *Arabidopsis thaliana 9-CIS-EPOXYCAROTENOID DIOXYGENASE 1 (NCED1)*, and an increase in *VvCYP707A*, a homologue of *A. thaliana CYTOCHROME P450 (CYP707A)*, which are implicated in ABA biosynthesis and catabolism, respectively (Zheng *et al.*, 2015; Wang *et al.*, 2016). In sweet cherry, we found that high ABA levels are correlated with deep dormancy whereas a decrease in ABA is correlated with dormancy release and bud break (Chapter 8). OKEN treatment caused a decrease in ABA level in flower buds, thereby overcoming endodormancy and initiating bud break. Another similarity between Dormex® and OKEN is a rapid increase in GA7 level after HC treatment (Wang *et al.*, 2016), similar to that observed after OKEN treatment ([Figure 9.9 B](#)), followed by a decrease in GA levels ([Figure 9.9 A, B](#)), as observed after HC treatment in grapevine buds (Zheng *et al.*, 2018). An increase in JA levels associated with increased JA biosynthesis-related gene expression was reported in sweet cherry after HC treatment (Ionescu *et al.*, 2017). On the other hand, we reported a marked decrease in JA levels after OKEN treatment. JA and its metabolites play a role in the regulation of plant growth and development, such as senescence, reproduction, and pollen and embryo development (Wasternack and Parthier, 1997; Balbi and Devoto 2008; Wasternack and Kombrink 2009). It has been shown that exogenous JA treatment can inhibit seed germination in several plants (Norastehnia *et al.* 2007; Miersch *et al.* 2008; Oh *et al.* 2009; Zalewski *et al.* 2010). So we can hypothesise that a decrease in JA triggered by OKEN might activate bud growth.

In simple terms, OKEN acts on dormancy release by decreasing ABA and GA9 levels and *PavDAM4* expression, while it increases the expression of *PavFT*, the level of GA7 and the oxidative stress status (GSSG/GSH ratio) within sweet cherry floral buds ([Figure 9.11](#)).

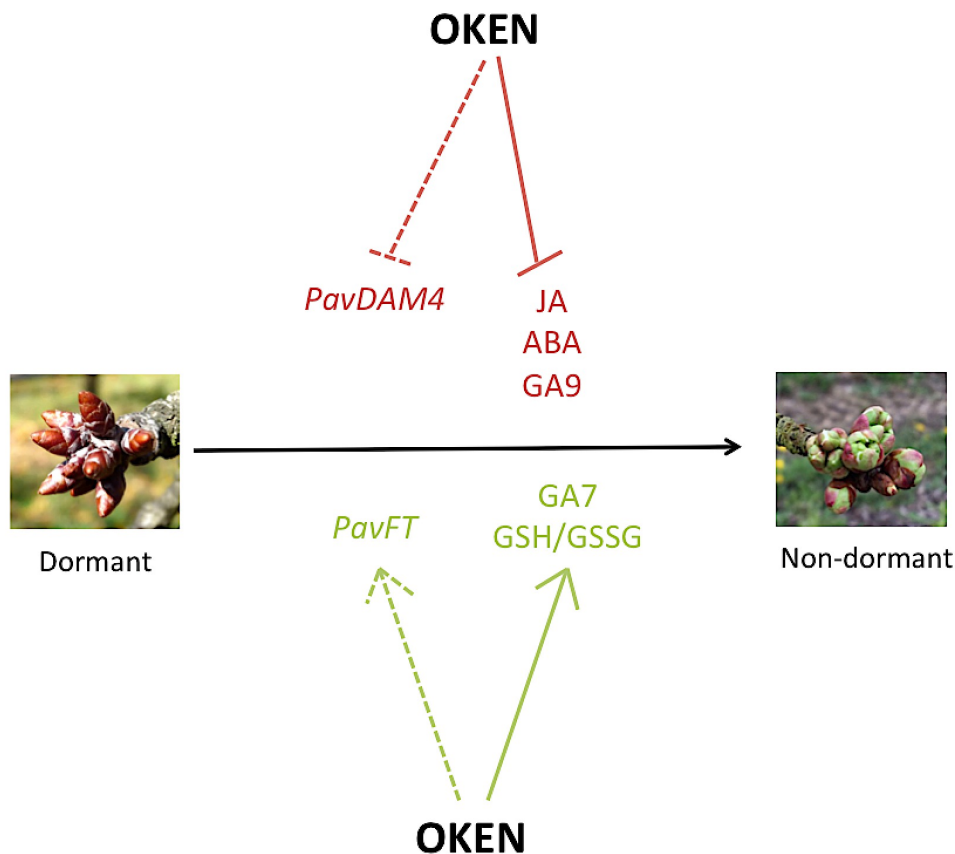


Figure 9.11 | Proposed mode of action for the OKEN-induced acceleration of budbreak in sweet cherry
 Red arrows indicate repressive action of OKEN at the gene level (dashed lines) and at the metabolite level (solid lines). Green arrows indicate active action of OKEN at the gene level (dashed lines) and at the metabolite level (solid lines). *DORMANCY ASSOCIATED MADS BOX 4* (*PavDAM4*); *FLOWERING LOCUS T* (*PavFT*); GA=gibbellic acid; ABA=abscissic acid and JA= jasmonic acid.

9.4.2 Contrary to Dormex®, OKEN presents no risk for buds when applied at the wrong time-interval

Dormex® (or HC) was reported to be extremely effective for breaking dormancy of vegetative buds but floral buds are less resistant to the toxic effects of HC (Or *et al.*, 1999). An acceleration of bud break was reported for several species after a HC treatment from two days to nine days in advance (Rahemi and Asghari, 2004; Son and Kuden, 2004; Sagredo *et al.*, 2005; Bound and Jones, 2010; Gaaliche *et al.*, 2016; Wang *et al.*, 2016). In addition, HC treatment had no effect on fruit size (Bound and Jones, 2010; Gaaliche *et al.*, 2016).

Cut branches and field assays have demonstrated that OKEN also has an impact on dormancy release and that two applications of a medium concentration (0.550%) is more efficient than one application at 1.1% (Figure 9.1 A). However, the efficiency of OKEN in overcoming endodormancy is dependent on the timing of application. OKEN displayed more rapid and higher percentage of bud break when applied on trees after the three quarter of chilling requirements (Date 2; Figure 9.5 B and Figure 9.6 B). Nonetheless, when OKEN was applied on cut branches over the different years of experiment, probably with different chilling

requirements and accumulation, buds always displayed higher percentage of bud break than Dormex® (Figure 9.1), for which wrong time-interval application led to bud necrosis, low burst rate and high bud drop (Figure 9.1 A, C, D). These observations for Dormex® were also confirmed in peach where the application of HC during the later stages of endodormancy induces high bud break, whereas HC treatments applied during the ecodormancy stage inhibited and delayed bud break but also damaged buds and stems (Siller-Cepeda *et al.*, 1992). In addition, it was also confirmed in field in 2017 that OKEN did not display a lethal effect on flower buds even if cold temperature to overcome endodormancy was not lacking (Figure 9.2 and Figure 9.3 A). Interestingly, trees treated with OKEN in 2018 did not show a decrease of bud break percentage over time compared to surfactant (Figure 9.5) suggesting that OKEN might protect buds against frost and more largely against extreme temperature changes triggered by climate change.

9.5 Conclusion

Regarding all positive effects of OKEN on dormancy release and protection against frost, this biostimulant seems to be a good alternative to replace Dormex®, as the latter is dangerous for human and animal health. In addition, OKEN is simple to use for growers, as no lethal effects are observed if the product is applied at the wrong time-interval. Additional tests must be conducted in order to estimate the optimal dates of application in sweet cherry, and also in other species, as OKEN did not show a dormancy breaking effect on apple buds, probably due to wrong timing of application. New tests should be performed in combination with accurate phenology models in order to adjust the date of application over years in function of regions, species and temperature. To further increase the effect of OKEN, a combination of OKEN with another biostimulant might be effective similarly to reports showing enhanced effects of Dormex® in combination with ethephon (ethylene precursor) (Bound and Jones, 2010).

10. General discussion

As sessile organisms, plants have the necessity to adapt to fluctuating environmental conditions. The ability to perceive and respond to changes in ambient temperature is one of the key determinants of their survival. This is particularly important for perennials in temperate climates that have to withstand large seasonal temperatures. In order to survive winter months, deciduous trees enter a state of dormancy that minimises energy expenditure, a process that needs to be carefully regulated to ensure that growth resumes only when favourable conditions return. Mild winter temperatures may prevent dormancy release, while extreme low temperatures may damage emergent reproductive or fruiting structures. These phenomena cause losses of billions of euros in the European fruit industry (Snyder & de Melo-Abreu, 2005; Canellas et al., 2017). Given current global climatic trends and the sensitivity of such plants to ambient temperature, it is of agricultural importance to understand the mechanisms by which dormancy is regulated in deciduous trees in order to sustain fruit productivity.

Several studies have demonstrated that the onset of dormancy in sweet cherry (*Prunus avium* L.) is triggered by low temperatures (Heide *et al.*, 2008) whereas dormancy release is regulated by warm temperatures, unlike many other perennials, whose dormancy cycle is dependent more on changes in photoperiod (Heide 2008 and 2011). Sweet cherry is therefore a useful model to study the mechanisms by which temperature signals are integrated into dormancy control. Using high-throughput analyses of gene expression, histone modifications and metabolites, this thesis has characterised the molecular events occurring during the dormancy cycle. What sets this work apart from the rest is the temporal resolution with which these analyses have been performed: floral buds were harvested during their early development in summer, through the para-, endo- and ecodormancy phases over autumn and winter, and on to bud break and beginning of flowering in spring. Gene expression was analysed by RNA-seq over ten/eleven dates from July through to March in four different varieties of sweet cherry with strikingly different patterns of dormancy release. In perspective, previous studies have used at most five harvesting dates in a single cultivar, for example in *Populus* axillary buds (Howe *et al.*, 2015), or have encompassed up to ten varieties but with a single harvesting date, for example in dormant *Prunus persica* floral buds (Leida *et al.*, 2012b). These

studies typically monitored gene expression using microarrays, which are associated with lower genome coverage and higher noise relative to RNA-seq.

Owing to its high varietal and temporal resolution, our RNA-seq dataset has highlighted a key feature of the dormancy cycle that previous studies failed to capture. It was previously suggested that differences in dormancy release between early- and late-flowering varieties were due to molecular events at the end of endodormancy. However, the RNA-seq analyses show clear differences in the transcriptomes of early- and late-flowering varieties already at the beginning of endodormancy, providing strong evidence that the depth of dormancy and the timing of dormancy release are determined early on. In addition to this discovery, we found that the various stages of dormancy are associated with distinct transcriptomic changes. This finding has allowed us to isolate marker genes that define the state of dormancy in buds and that will be invaluable for monitoring bud dormancy in the field. In the future, it will be of interest to test these marker genes in additional varieties of sweet cherry and in other perennial species of agricultural importance. In addition, given the apparent importance of molecular events at the beginning of endodormancy in controlling the transition from dormancy to bud break, it will be essential to complement our RNA-seq dataset with a higher-resolution analysis of gene expression changes occurring during the early stages of endodormancy. Thanks to our RNA-seq data, we found that the ecodormancy phase is marked by a recovery of cellular activity, indicative of bud-break. In addition, we observed increased oxidative stress in buds during dormancy release triggered by the biostimulant (OKEN). This reinforces the team hypothesis' that the combination of abiotic stress and a decrease in cell metabolism during dormancy leads to an accumulation of ROS, whereas dormancy release is associated with ROS scavenging and detoxification (Beauvieux *et al.*, 2017 and 2018; [Figure 10.1](#)).

Genes encoding transcriptional regulators of the *Dormancy-Associated MADS-box (DAM)* family have been of special interest in the last decade because of their involvement in the regulation of dormancy, as epitomised by the peach *evergrowing (evg)* mutant, containing a deletion of six *DAM* genes and characterised by the inability to enter dormancy (Bielenberg *et al.*, 2004 and 2008). *DAM* genes have been the focus of numerous studies that investigated their expression patterns and interactions in heterologous expression assays (Horvath *et al.*, 2010; Sasaki *et al.*, 2011; Saito *et al.*, 2015; Tuan *et al.*, 2017; Zhao *et al.*, 2018). Given the time associated with generating transgenic perennials and the current inability to transform sweet cherry, little is known about the activity of sweet cherry PavDAM transcription factors. To address this issue, an *in vitro* chromatin immunoprecipitation

(ChIP) method was developed and the potential genome-wide binding sites of sweet cherry PavDAM proteins were investigated. Because they contain a repressive EAR motif in their C-terminus, DAM proteins have been hypothesised to act as transcriptional repressors. Interestingly, PavDAM6 was found to act as a transcriptional activator in yeast, raising the question as to whether it may possess such ability in sweet cherry as well. In the future in order to test this hypothesis and to better understand the role of *PavDAM* during dormancy, it will be key to confirm PavDAM binding sites and transcriptional activation or repression *in vivo*, by developing an *Agrobacterium*-mediated transformation procedure for sweet cherry calli, as has been published for apple (An *et al.* 2018). It will be essential to generate null mutants for *DAM* genes in the sweet cherry using RNA interference or the CRISPR/Cas9 system, including deletions of multiple genes, to generate lines similar to the *Prunus persica evg* mutant and thereby confirm the importance of DAMs in the sweet cherry, and also deletions of individual genes, in order to study the importance of the different DAMs in the dormancy cycle.

It will also be key to perform mutational or over-expression analyses on candidate genes identified from the RNA-seq, with the aim of identifying key regulators of endo- and eco-dormancy, ultimately allowing for the generation of new cultivars with a short endodormancy phase to withstand limited chill units during mild winters or with an extended ecodormancy phase to prevent the requirement for warm units being satisfied too rapidly. The RNA-seq dataset produced in this thesis is useful material to study transcription factors implicated in dormancy control. In the future, it will be of interest to study the binding sites of transcription factors whose expression patterns correlate with the dormancy cycle, as determined from our RNA-seq dataset. In addition, the promoters of the various clusters of genes whose expression changes over the dormancy cycle will be analysed for conserved motifs, which together with *in vitro* ChIP-seq might allow us to generate a map of the regulatory network of key gene expression events occurring during dormancy.

It would be interesting to carry out RNA-seq on twigs treated with hormones implicated in dormancy release, such as ABA and GA, and to compare transcriptomic changes during such treatments with those observed from the RNA-seq performed on trees in the field during the course of the dormancy cycle. Furthermore, the RNA-seq analyses of this thesis could be extended to study miRNAs and siRNAs, as a growing number of publications have demonstrated that these RNAs are implicated in dormancy in numerous perennials (Barakat *et al.*, 2012; Ding *et al.*, 2014 and 2016; Cui *et al.*, 2016; Guo *et al.*, 2017; Zhang *et al.*, 2018).

An additional exciting experiment would be to grow potted trees under controlled temperature conditions and to see whether these plants eventually enter a dormant state when exposed to extended periods of summer-like conditions (warm temperature and long-day photoperiod) without a dormancy signal (lower temperature and short-day photoperiod), which would suggest that an innate clock- or memory-based trigger for dormancy. If this is the case, as certain personal observations indicate (Benedicte Wenden, unpublished results), RNA-seq analyses similar to those performed in this thesis may be used to characterise the transcriptomic differences between these two types of dormancy.

Another interesting observation from the dormancy-associated transcriptome was that the expression of histone modifier genes increases during endodormancy release and bud break, suggesting that the dormancy cycle is under epigenetic control. Epigenetic analyses using ChIP-seq of post-translationally modified histones demonstrated that there are no major changes in histone H3 lysine-4 trimethylation (H3K4me3) during the course of dormancy or bud break. It is possible that this was a result of the final harvesting date being too early in the ecodormancy phase, again highlighting the importance of greater temporal resolution during such analyses. Histone H3 lysine-27 trimethylation (H3K27me3) was detected at specific genomic loci and, in contrast to H3K4me3, was found to increase during dormancy (Figure 10.1). A more thorough analysis of this ChIP-seq will allow us to connect changes in chromatin marks and gene expression during dormancy. The RNA-seq and ChIP-seq datasets generated in this thesis are currently limited by the availability of the sweet cherry genome, a preliminary assembly of which has only recently been published (Shirasawa et al., 2017). ChIP-seq and RNA-seq reads were therefore mapped onto this sweet cherry genome or onto the well-annotated genome of the cherry relative, *Prunus persica*. In the future, it will be essential to improve the quality and annotation of the sweet cherry genome. This will allow for more in-depth epigenetic analyses, potentially on a greater diversity of chromatin marks. Before performing additional ChIP-seq, it will be essential to conduct trials using ChIP-qPCR to ensure that the harvesting dates provide optimal resolution.

In addition to providing an insight into transcriptional and epigenetic regulation of dormancy in sweet cherry, we performed highly sensitive measurements of metabolites over eleven sampling dates encompassing the dormancy cycle, including major phytohormones as well as their precursors and catabolites. In accordance with previous studies, we found a correlation between the abundance of abscisic acid (ABA) levels and the delay in bud burst and flowering. This is corroborated by our RNA-seq dataset, in which we observed contrasted expression patterns for genes

involved in ABA biosynthesis and catabolism between cultivars. In particular, we identified the enhanced expression of a specific UDP-glycosyltransferase (*PavUGT71B6*), which induces lower levels of ABA in the early-flowering cultivar of sweet cherry. We also detected dormancy-associated fluctuations in the levels of various gibberellins and this study revealed a complex regulation of gibberellin biosynthetic and catabolic genes at a level downstream of transcription, as the expression of such genes did not correlate with gibberellin levels (Figure 10.1).

Finally, in collaboration with the Roullier Group, a biostimulant identified as OKEN was shown to accelerate dormancy release. The most widely used agent to stimulate bud break has traditionally been hydrogen cyanamide (commercialised as Dormex[®]), however because of its acute toxicity to human health it is now banned in many countries across the world. We have demonstrated that OKEN acts via a similar pathway as hydrogen cyanamide, inducing pathways associated with redox regulation and floral transition. In addition, OKEN may have a stronger effect on dormancy release than hydrogen cyanamide, and thus it might be a useful alternative to Dormex[®]. In the future, it will be necessary to perform a time-series treatment of OKEN on buds from various varieties of sweet cherry in order to evaluate the optimal application period. The marker genes identified in this thesis may be used to predict the state of dormancy during treatments with OKEN, thereby validating their use in monitoring bud dormancy in the field. In addition, it will be of interest to test whether OKEN has similar effects on dormancy release in other perennial species. OKEN, combined with an expression-based method for predicting dormancy release, would be an invaluable resource for agriculture.

In the long-term, it will be essential to develop new varieties of fruit trees better adapted to the changing climate: low chill requirements associated with advanced dormancy release dates, under mild winter conditions, potentially coupled with dormancy-breaking reagents, like OKEN and hydrogen cyanamide, to promote endodormancy release. One of the varieties studied in this thesis, 'Cristobalina', is characterised by low levels of ABA and extremely early endodormancy release and flowering dates, therefore with very low chill requirements, suggesting that it may be of potential use for enhancing cherry production in the future. From the RNA-seq dataset generated in this thesis, *PavDAM4* expression was found to be lowest in 'Cristobalina' and highest in the late-flowering cultivar 'Regina', with endodormancy release correlating with a reduction in *PavDAM4* expression. *PavDAM4* or ABA biosynthetic or catabolic genes could therefore potentially be used as marker genes for breeding new cultivars better suited to climatic changes, using marker-assisted selection. Trees presenting low ABA level and low *PavDAM4* expression during

dormancy could be used as parents to generate new low-chilling cultivars adapted to increased winter temperatures. Additionally, it will also be necessary to further manipulate, and more specifically extend, ecodormancy to prevent premature bud break under warmer spring temperatures. In this study, we have mainly focused on mechanisms controlling endodormancy but data are now available to further investigate the ecodormancy phase in contrasted cultivars. In conclusion, this thesis has provided crucial insight into dormancy regulation in perennials and has additionally presented a commercial solution to sustain fruit production despite an ever-changing climate.

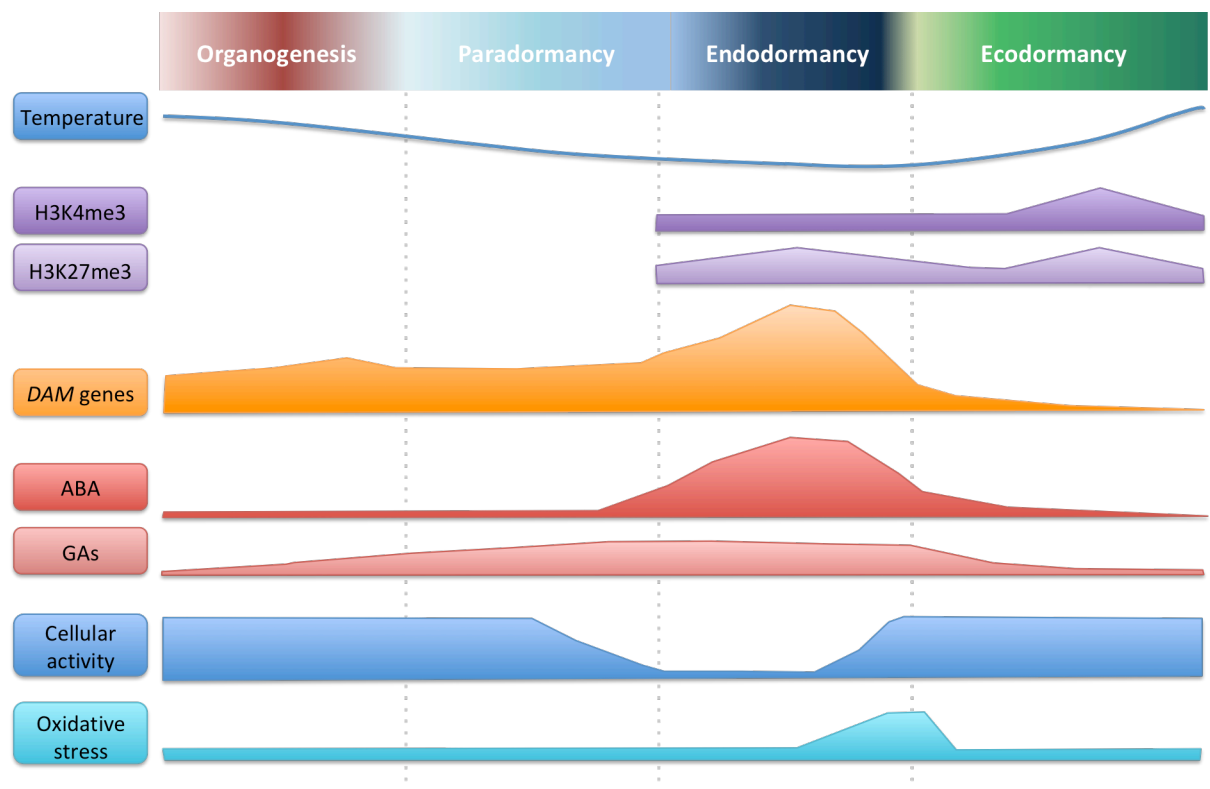


Figure 10.1 | Summary of key findings from this PhD thesis

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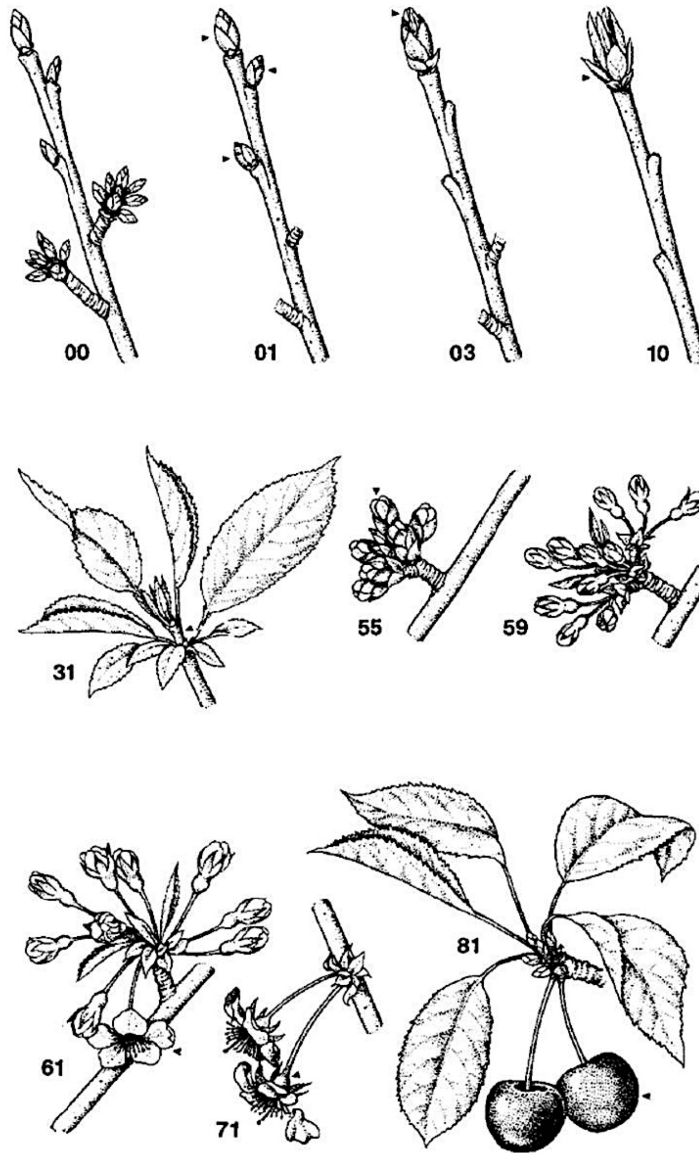
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Appendix

BBCH code	Description
Principal growth Stage 0: bud development	
00	Dormancy
01	Beginning bud swelling
03	End of leaf bud swelling
09	Green leaf tips visible
Principal growth Stage 1: leaf development	
10	First leaves separating
11	First leaves unfolded
19	First leaves fully expanded
Principal growth Stage 3: shoot development	
31	Beginning of shoot growth
32	20% of final shoots length
33	30% of final shoots length
3...	Stages continuous till...
39	90% of final shoots length
Principal growth Stage 5: reproductive development or inflorescence emergence	
50	Dormancy, inflorescence bud closed
51	Inflorescence buds swelling
53	Bud burst
54	Inflorescence enclosed by light green scales
55	Single flower buds visible
56	Flower pedicel elongating
57	Sepals open
59	Balloon
Principal growth Stage 6: flowering	
60	First flowers open
61	Beginning of flowering
62	20% of flowers open
63	30% of flowers open
64	40% of flowers open
65	Full flowering
67	Flower fading
69	End of flowering
Principal growth Stage 7: fruit development	
71	Ovary growing
72	Sepals beginning to fall
73	Second fruit fall
75	50% of final fruit size
76	60% of final fruit size
77	70% of final fruit size
78	80% of final fruit size
79	90% of final fruit size
Principal growth Stage 8: ripening or maturity	
81	Beginning of fruit colouring
85	Colouring advanced
87	Fruit ripe for picking
Principal growth Stage 9: senescence, beginning of dormancy	
91	Shoot growth completed; foliage still fully green
92	Leaves begin to discolour
93	Beginning of leaf fall
95	50% of leaves fallen
97	All leaves fallen

Appendix Figure 1 | Phenological growth stages of sweet cherry according to the BBCH scale (Fadón *et al.*, 2015).

Stone fruit



© 1994: BBA und IVA

Appendix Figure 2. | BBCH scales according to Meier *et al.*, 1994

IG	S genotype	Cultivars
I	S_1S_2	'Black Tartarian', Canada Giant™ ('Sumgita'), 'Early Rivers', 'Ferdouce', 'Starking Hardy Giant', 'Summit'
II	S_1S_3	'Black Star', Cristalina™ ('Sumnue'), 'Early Van Compact', 'Giant Red' (Giant Ruby™), 'Lala Star', 'Prime Giant', 'Regina', Samba™ ('Sumste'), Satin™ ('Sumele'), 'Sonnet' ('Sumbola'), 'Van', 'Vera', 'Windsor'
III	S_3S_4	'Belge', 'Bing', 'Lambert', 'Napoleon' ('Royal Ann'), 'Somerset', 'Star', 'Ulster'
IV	S_2S_3	'Nimba', 'Coralise' ('Gardel'), 'Sue', 'Vega'
VI	S_3S_6	'Ambrunés', 'Anita', 'Badacsony', 'Fertille', 'Kordia', 'Pico Negro', 'Satonishiki', 'Stark Gold' ('Dönissens Gelbe', 'Gold'), 'Techlovan', 'Duro 3', 'Ferdiva', 'Fertard'
VII	S_3S_5	'Hedelfinger'
IX	S_1S_4	'Black Giant', 'Black Republican', 'Garnet', 'Hudson', 'King Rainier', 'Salmo', 'Sylvia'
X	S_6S_9	'Folfer', 'Ramón Oliva'
XIII	S_2S_4	'Corum', 'Deacon', 'Patricia', 'Peggy Rivers', 'Royalton', 'Sam', 'Schmidt', 'Vic'
XIV	S_1S_5	'Alma', 'Annabella', 'Blanca de Provenza', 'Seneca', 'Valera'
XV	S_5S_6	'Colney'
XVI	S_3S_9	'Burlat', 'Chelan', 'Moreau', 'Precoce Bernard', 'Tieton'
XVII	S_4S_6	'Larian', 'Merton Glory'
XVIII	S_1S_9	'Bigisol' ('Early Bigi'), 'Brooks', 'Earlise®Rivedel' ('Rivedel'), 'Early Red' ('Early Garnet'), 'Rocket', 'Marvin' ('Niran', '4-70'), 'Sweet Early' ('Panaro 1)'
XIX	S_3S_{13}	'Reverchon'
XX	S_1S_6	'Vanda'
XXI	S_4S_9	'Cashmere', 'Merchant', 'Merpet'
XXII	S_3S_{12}	'0900-Ziraat', 'Ferrovía', 'Germersdorfi', 'Noire de Meched Schneiders'
XXIV	S_6S_{12}	'Aida', 'Flementier'
XXV	S_2S_6	'Fercer' ('Arcina')
XXVI	S_5S_{13}	'Ferbolus' ('Verdel'), 'Goodnestone Black'
XXVII	S_4S_{12}	'Margit', 'Kavics'
XXXIII	S_1S_{14}	'Fermina'
XLIII	S_2S_9	'Primulat' ('Ferprime')
SC ^a	S_1S_4	Celeste™ ('Sumpaca'), 'Frisco', 'Lapins', 'Santina', 'Skeena', 'Symphony', 'Stardust'
	S_3S_4	'Compact Stella', 'Index', 'Newstar', 'Sandra Rose', 'Selah' ('Liberty Bell'), Sonata™ ('Sumleta'), Staccato™ ('Summer Charm', '13S2009'), 'Starkimson', 'Stella', 'Sumesi', 'Sunburst', Sweetheart™ ('Sumtare'), 'Tehranivee'
	S_3S_3	'Axel'
	S_3S_6	'Cristobalina', 'Temprana de Sot'
	S_4S_6	'Blaze Star', 'Blackgold'
	S_4S_9	'Early Star' ('Panaro 2), 'Pacific Red', 'Sandor', 'Columbia' ('Benton'), 'Grace Star', 'Glacier'
	S_5S_6	'Kronio'
0	S_5S_{22}	'Rita'

^aAll SC cultivars included are also universal donors.

Annex Table 3 | Incompatibility groups (IGs) of selected sweet cherry cultivars.

From Schuster, 2012; Cachi *et al.*, 2015 in Herrero *et al.*, 2017

	PAV_r1.0
Estimated genome size (bp)	352,883,670
# of scaffolds	10,148
Size of scaffolds (bp)	272,361,615
Scaffold N50 (bp)	219,566
Longest scaffold (bp)	1,460,269
GC (%)	37.7
# of genes	43,673
Mean size of genes (bp)	1,097
Repeat (%)	43.8

Appendix Table 4 | Sequencing data of the sweet cherry genome ‘Stella’

	‘Regina’	‘Satonishiki’ (Shirasawa et al, 2017)
Technology	PacBio RSII + BioNano	Illumina HiSeq
Number of scaffolds	92	10,148
Size of scaffolds (bp)	279 Mb (hybrid scaffold)	272 Mb
Scaffold N50 (bp)	5.96 Mb	0.21 Mb
Longest scaffold (bp)	16.3 Mb	1.46 Mb
GC (%)	38.2	37.7

Appendix Table 5 | Comparison of the two sweet cherry genomes ‘Regina’ and ‘Satonishiki’ sequences (Dirlewanger et al., 2018)

Technology	Tiling microarray	cDNA or EST sequencing	RNA-Seq
Technology specifications			
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	From several to 100 bp	Single base	Single base
Throughput	High	Low	High
Reliance on genomic sequence	Yes	No	In some cases
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited for gene expression	Yes
Dynamic range to quantify gene expression level	Up to a few-hundredfold	Not practical	>8,000-fold
Ability to distinguish different isoforms	Limited	Yes	Yes
Ability to distinguish allelic expression	Limited	Yes	Yes
Practical issues			
Required amount of RNA	High	High	Low
Cost for mapping transcriptomes of large genomes	High	High	Relatively low

Appendix Table 6 | A list of advantages of RNA-seq compared with other transcriptomic methods. (Wang et al., 2009).

		Supplier	Catalogue number	Stock solvent	Storage
HDAC inhibitor	Nicotinamide	Sigma	N3376	milliQ water	Filtration and TA
	Trichostatin A	Sigma	T8552	DMSO	-20°C
	Vorinostat (SAHA)	Sigma	S8690	DMSO	-20°C
	Sodium butyrate	Sigma	B5887	milliQ water	-20°C
HAT inhibitor	Anacardic acid	Sigma	A7236	DMSO	-20°C
	Curcumin	Sigma	C1386	Ethanol absolute	-20°C
DNMT inhibitor	5-azacytidine	Sigma	A2385	DMSO	-20°C

Appendix Table 7 | Epigenetic drugs used in this study

HDAC: histone deacetylase, HAT: histone acetyltransferase and DNMT: DNA methyltransferase

Primer number	Sequence (5'→ 3')	Purpose
7605	CTTCAAAGGCTCGGTTGT	<i>SSA1</i> (yeast gene as control)
6977	AGATACATCAATCCACCCCTTA	
9306	TGGCGAGGGAGAAGATTCAG	<i>DAM2</i> , exon 1
9307	TGGAGCTGGCGTATTCAAAG	
9308	CTGGAGAAACAAAGTAGCCG	<i>DAM2</i> , exon 7
9309	GCAGAGGTTGGTGGTGACAG	
9318	TGGCGAGGGAGAAGATTCAG	<i>SVP</i> , exon 1
9319	TGGAGCTGGCGTATTCAAAG	
9784	CACATCAACATCGTGGTCA	<i>EF1</i> , positive control for H3K4me3
9785	GAACCTCTCAATGACACGC	
9793	GAAGATTGACTACTTGCCTGC	<i>DAM5</i> , exon 1
9794	AGAAAAGATGACAACCTGCCAC	
9795	TGAAGGGCTGAATCTGGA	<i>DAM5</i> , exon 4
9796	TAGTTTCCATGACACGGC	
9797	GGAATACTGGACCTGCG	<i>DAM5</i> , exon 7
9798	GCCTTCTTCTTCTCCTCCA	
9799	TGGAGGAGAAGAAGAAGGC	<i>DAM5</i> , exon 7
9800	TCATCGGAGGAGTCATCTTC	
12600	TTGTTCCATACACAGTAGCAT	<i>UBIQUITIN</i>
12601	GTCTGATACCGTCATTGCTTA	
12602	AATGGTGACCCTCTTAGCG	<i>H3</i>
12603	CTACTTGGTGGGTCTGTTGA	
12604	AGCAAAGCAAGCAAGAATGT	<i>RPII</i>
12605	TCTGAAATGGAAGGCAA	
12621	CGACAAGCAACCTATCCC	<i>Ga3ox</i>
12622	GTTCGGACAGAGCCATAGT	
12625	ACCGACCCACTTTCTCAG	<i>NAC5</i>
12626	CTTCGTCTGTGGGATAGA	
12631	GACATTTCAAACAGAACAACA	<i>GRF7</i>
12632	TGAAGGCGGTATGATGAG	
12633	GTGTTAGCAGGAATACCAGAAG	<i>SEX1</i>
12634	CCAAGAGTGTCACCAAGT	
13194	AGGTGTTGGACAAGTATGAGG	<i>AG</i> , positive control for H3K27me3
13195	TAAGGGGCTGACGAGAG	
13196	CAAGAAGATTGACTACCTGCC	<i>DAM4</i>
13197	TCCCTCTTCTCCTTTTGA	
13198	TCAAAGAGGAGAAGAGGGA	<i>DAM4</i>
13199	TGCCACCTCAGATTCACA	
13225	CTCAAAGAGAAGAAGAGGGCT	<i>DAM6</i>
13226	CACCTCAGATTCACACAGAAC	
13227	GGACCCGCTTGTTGTT	<i>FT</i>
13228	GTAAACGGGTCTAAAACATCAC	

Appendix Table 8 | Primers used in qPCR

Primer number	Sequence (5'-> 3')	Purpose
11025	GAACGCAGACCCCGAACTCACCAACGAAGGT	Amplification of <i>DAM5</i> and <i>DAM6</i>
11026	CCCCAGTTTGAGGGATAAAGTGACCTCATCGGAGG	
11027	TTTTCTGGACAGACCAAACTTCAGACCGGGCT	
11028	CCTTGTCACCTTCTTAAGTAGGAACTAGGGAAGC	
13118	CCAAGCATACAATCAACTCCAAGCTTATGGATTATAAAGATGACGATG ACAAGGATTATAAAGATGACGATGACAAGGATTATAAAGATGACGATG ACAAG	Amplification of N-FLAG and C-FLAG
13119	GCGGCCGTTACTTACTTAGAGCTCAGCCTTACTAACAAGCTAGCCTTGTCAT TCGTCATCTTTATAATCCTTGTGCATCGTCATCTTTATAATCCTTGTGCAT CGTCATCTTTATAATC	
13120	CCAAGCATACAATCAACTCCAAGCTTGAAGCTCGCTAGTGTAGTAAGGC TGATTATAAAGATGACGATGACAAGGATTATAAAGATGACGATGACAA GGATTATAAAGATGACGATGACAAG	
13121	GCGGCCGTTACTTACTTAGAGTCACTTGTGCATCGTCATCTTTATAATCC TTGCATCGTCATCTTTATAATCCTTGTGCATCGTCATCTTTATAATC	
7066	CCTTCATTCACGCACACTACTCTC	Genotyping and sequencing of pDBleu-NFLAG and pDBleu-CFLAG
6161	TTTGACCCTTTTCCATCTTTTCGTAA	
13138	AGGCTAGTGTTAGTAAGGCTGAGATGATGAGGGAGAAGATCAAGA	Amplification of <i>pDBleu::FLAGx3::gene</i>
13139	GCGGCCGTTACTTACTTAGAGTCACCCTAATTTGAGAGATAAAATG	
13140	AGGCTAGTGTTAGTAAGGCTGAGATGCCTAGGGACAGGGA	
13141	GCGGCCGTTACTTACTTAGAGTTATCTTCTCCTTCTCCTCCGG	
13134	AGGCTAGTGTTAGTAAGGCTGAGATGATGAGGAATAAGATCAAGATCA	
13135	GCGGCCGTTACTTACTTAGAGTTAAGTCCCAGTTTGAGGGATAAA	
13136	AGGCTAGTGTTAGTAAGGCTGAGATGATGAGGGAAAAGATCAAAA	
13137	GCGGCCGTTACTTACTTAGAGCTAGGGAAGCCCAATTTG	
13144	CAATCAACTCCAAGCTTGAAGATGATGAGGGAGAAGATCAAGA	
13145	CAGCCTTACTAACAAGCTAGCGAGTACCCTAATTTGAGAGATAAAATG	
13146	CAATCAACTCCAAGCTTGAAGATGCCTAGGGACAGGGA	Amplification of <i>pDBleu::gene::FLAGx3</i>
13147	CAGCCTTACTAACAAGCTAGCGAGTTATCTTCTCCTTCTCCTCCGG	
13148	CAATCAACTCCAAGCTTGAAGATGATGAGGAATAAGATCAAGATCA	
13149	CAGCCTTACTAACAAGCTAGCGAGTTAAGTCCCAGTTTGAGGGATAAA	
13150	CAATCAACTCCAAGCTTGAAGATGATGAGGGAAAAGATCAAAA	
13151	CAGCCTTACTAACAAGCTAGCGAGCTAGGGAAGCCCAATTTG	

Appendix Table 9 | Primers used in *in vitro* ChIP assay

>PavDAM4

ATGATGAGGGAGAAGATCAAGATCAAGAAGATTGACTACCTGCCAGCACGGCAGGTGACCTTCTCAAAGAG
GAGAAGAGGGATCTTCAAGAAAGCTGCAGAGCTATCTGTTCTGTGTGAATCTGAGGTGGCAGTTGTCATCTT
TTCTGCTACTGGGAAGCTTTTTGATTATTCGAGCTCAAGCGTCAAGGATGTAATTGAAAGGTACAAAGCGCGC
ACAAATGGTGTGCAAAAATCGGACAAGTCTCTTGAGCTGCAGCTGGAGAATGAAAACCGCATCAAATGAGT
AAGGAACTCGAGGAGAAGAACC GCCAGCTGAGGAAGATGAAAGGTGAGGATCTTGAAGAGCTGGATCTGGA
TGAGTTGCTGAAGTTGGAACAACCTGGTGGAAAGCAACCCTTGTCCGTGTGATGGAAACTAAGGAAGAAGT
TATGAGTGATATTATGGTATTGGAGAAAAGGGAAGTGAAGTGGTAGAAGCCAACAATCAGATGGTGTGTT
AAAGGAGAGGATGGTTATGTTATCCAAAAGAAATACTGAACCTGCGCATATGGAGCCATCTGAATCTGCTAC
CTCCACCAGCTGCAACAGTGTCTCAGTCTCTCTGGTGAAGATGACTGCTCCGACGACGTCATTTTATCTCT
CAAATTAGGGTGA

> PavDAM5

ATGATGAGGAATAAGATCAAGATCAAGAAGATTGACTACTTGCCTGCAAGGCAGGTGACCTTCTAAAAAGG
AGAAGAGGGGCTCTTCAAGAAAGCTGCAGAGCTATCTGTTCTGTGTGAATCTGAGGTGGCAGTTGTCATCTTT
TCTGCTACTGGCAAGCTTTTCGATTATTCAGTTCAAGTACCAAGGATGTTATTGAAAAATACAACGTGCACAT
GAATGGTGTGCAAAAATGGAATGATCAAGAGATTGAACTGCAGCTGGAGCATGAAAACCATCAAATGAGT
TAAGGAACTCGAGGAGAAGAGCCGCCAGCTGAGGCAGATGAAAGGTGATGATCTTGAAGGGCTGAATCTGG
ATGAGTTGCTGAAGTTGGAACAACCTGGTGGAAAGCAAGCCTTGGCCGTGTGATGGAAACTAAGGAAGAGCTG
ATTAAGAGTGAGATTATGGCACTTGAAGAAAAGGGAGCTGAGCTAGTTGAAGCCAACAACAGCTAAGGCAG
ACGATGGTGTGTTATCTGCAGGAAATACTGGACCTGCGCTTATGGATCCGGAGAGGTTGAACAATAATATT
GAAGGTGGAGGAGAAGAAGAAGGCATGTCAGCTGAATCTGCTATCTCCACCACCTGCAACAGTGTCTGTCAG
TCTCTCTTTGAAGATGACTCCTCCGATGAGGTCACCTTATCCCTCAAACCTGGGGACTTAA

>PavDAM6

ATGATGAGGGAAAAGATCAAAAATCAAGAAGATTGACTACCTGCCAGCAAGGCAGGTTACCTTCTCAAAGAGA
AGAAGAGGGGCTCTTCAAGAAAGCTGCAGAGCTATCTGTTCTGTGTGAATCTGAGGTGGCAGTTGTCATATTTT
CTGCTACTGGCAAGCTCTTTGATTATTCAGCTCAAGTATCGAGGATGTTCTTGAAGGTACAAAGCGCACAC
AAATGGTGTGCAAAAATCGAACAACAGTTTCTTGAGCTGCAACTGGAGAACGAAAAACACATCAAATGAGT
AAGGAACTCGAGGAGAAGAGCCGCCAGCTGAGGCAGATGAAAGGTGAGGATCTTGAAGGGCTGAATCTGG
ATGAGCTGCTGAAGTTAGAACAACCTGGTGGAAAGGAAGCCTTGGCCGTGTGATAGAAAATAAGGAAGAGCTG
ATTATGAGTGAGATCATGTCACCTGGAGAAAAGGGAGCTGAGCTGGTAGAAACCAACAACAGTTAAGGCAG
AGGATGGCGATGTTATCCGGAGGAAATACTGGACCTGCGCTTGTGGAGCCGGAGACGTTGAATACTAATATT
GGAGGTGGAGGAGAAGACGGCATGTCATCTGAATCTGCCACAATGGCCACCTCTACCAGCTGCAATAGTGC
TCTCAGTCTCTCTTTGAAGATGACTGCTCCGATGTCACCTTATCTCTCAAATTGGGGCTTCCCTAG

>PavFT

ATGCCTAGGGACAGGGACCCGCTTGTGTTGGAAGAGTGGTAGGTGATGTTTTAGACCCGTTTACAAGGTCT
GTTTCTCTCAGGGTCACTTACGGTATGAAGGAGGTTAACAATGGTTGCGAGCTCAAACCTTCCCAAGTTGTC
CACCAACCTAGAGTTGATACTGGTGGGGATGATCTTAGGACTTTCTACACTCTGGTCATGGTGGATCCTGAT
GCACCTAGCCCAAGTGACCCCAACCTAAAGGAATATTTGCATTGGCTGGTTACGGATATCCAGCAACAAC
GCGGCAAGCTTTGGGCAAGAGATTGTGTGTTATGAAAGCCACGGCCAACGGTGGGGATTTCATCGCTTTGT
TTTGGTGTGTTTTCGGCAATTGGGTAGGCAAACAGTGTATGCTCCGGGATGGCGCCAGAACTTTAATACAAG
AGACTTTGCGGAGCTTTACAATCTTGGATTACCGGTAGCTGCCGTCTATTTTAATTGTCAAAGAGAAAGCGGC
TCCGGAGGAAGGAGAAGATAA

Appendix Figure 10 | CDS sequences of *PavDAM4*, *PavDAM5*, *PavDAM6* and *PavFT* in *Prunus avium* L.

Primer number	Sequence (5'-> 3')	Purpose	
13154	CCATGGAGGCCGAATTGATGATGAGGGAGAAGATCAAGA	Amplification	
13155	TGCAGGTCGACGGATCTCACCTAATTTGAGAGATAAAATG		
13156	CATGGAGGCCAGTGAATTGATGATGAGGGAGAAGATCAAGA		
13157	CTCGAGCTCGATGGATCTCACCTAATTTGAGAGATAAAATG		
13158	CCATGGAGGCCGAATTGATGATGAGGAATAAGATCAAGATCA		
13159	TGCAGGTCGACGGATCTTAAGTCCCAGTTTGAGGGATAAA		
13160	CATGGAGGCCAGTGAATTGATGATGAGGAATAAGATCAAGATCA		
13161	CTCGAGCTCGATGGATCTTAAGTCCCAGTTTGAGGGATAAA		
13162	CCATGGAGGCCGAATTGATGATGAGGGAAAAGATCAAAA		
13163	TGCAGGTCGACGGATCCTAGGGAAGCCCAATTTG		
13164	CATGGAGGCCAGTGAATTGATGATGAGGGAAAAGATCAAAA		
13165	CTCGAGCTCGATGGATCCTAGGGAAGCCCAATTTG		
13166	CCATGGAGGCCGAATTGATGCCTAGGGACAGGGA		
13167	TGCAGGTCGACGGATCTTATCTTCTCCTTCCTCCGG		
13168	CATGGAGGCCAGTGAATTGATGCCTAGGGACAGGGA		
13169	CTCGAGCTCGATGGATCTTATCTTCTCCTTCCTCCGG		
13221	CATCGGAAGAGAGTAGTAACAAAGGTC		Genotyping and sequencing
13222	ATGAAGATACCCACCAAACCC		
6483	AGGCTTACACTTTATGCTTCC		
6161	TTTGACCCTTTTCCATCTTTTCGTAA		

Appendix Table 11 | Primers used for the Yeast Two Hybrid assay

Primer number	Sequence (5'-> 3')	Purpose
13394	CCATGGAGGCCGAATTGATGCCAAAAAAGAAGAGAAAGGTCG	Amplification
13395	TGCAGGTCGACGGATCTCACTCTTTTTTTGGGTTTGGTG	
13397	TGCAGGTCGACGGATCTTATCTTCTCCTTCCTCCGG	
6161	TTTGACCCTTTTCCATCTTTTCGTAA	Genotyping and sequencing
13221	CATCGGAAGAGAGTAGTAACAAAGGTC	

Appendix Table 12 | Primers used for the Trans activation/repression assay

The presence of H3K4me3 histone mark is positively correlated with the expression of *DAM* genes in sweet cherry during dormancy

N. Vimont^{1,2,3}, R. Beauvieux¹, M. Arkoun², J.C. Yvin², E. Dirlewanger¹, S. Cortijo³, P.A. Wigge³ & B. Wenden¹

¹UMR 1332 BFP, INRA, Université de Bordeaux, F-33140 Villenave d'Ornon, France

²Agro Innovation International - Centre Mondial d'Innovation - Groupe Roullier, 35400 St Malo, France

³The Sainsbury Laboratory, University of Cambridge, CB2 1LR, United Kingdom

ABSTRACT

In perennial species such as sweet cherry, dormancy is a period of repressed growth that acts to protect sensitive tissues during unfavourable climatic conditions. Dormancy and bud burst are strongly affected by temperature. The mean surface temperature of the earth is increasing and this climatic change may have serious negative consequences on the dormancy release, potentially resulting in lower cherry production. Despite this strong effect of temperature, the molecular events regulating dormancy are still poorly understood. It has been shown that *DORMANCY-ASSOCIATED MADS-box (DAM)*-related genes are up-regulated in dormant buds and inversely down-regulated in non-dormant buds in several perennial plant species (peach, pear, leafy spurge, apple). Recent studies suggested the role of epigenetic mechanisms, such as DNA methylation and chromatin modifications, in the regulation of bud dormancy and dormancy release. To better understand the mechanisms underlying the effect of temperature on dormancy we assessed genome-wide dynamics for one chromatin mark (H3K4me3) associated with open chromatin and gene activation. It was coupled with expression during dormancy in floral buds of sweet cherry cultivar 'Burlat' (*Prunus avium* L.). We detected transcriptional and histone methylation dynamics of dormancy-related genes (*PavDAM5* and *PavDAM6*) during the dormancy cycle.

Keywords: *Prunus avium* L., chromatin immunoprecipitation, ChIP-seq, RNA-seq, transcriptomics, H3K4me3.

INTRODUCTION

Seasonal changes oblige plants to adapt their physiology to survive under unfavourable climatic conditions. Dormancy is one of these processes found in trees to persist under low winter temperature and short photoperiod (Faust et al., 1997), and thus allows the successful flowering establishment of perennial fruit species including cherry, peach (Yamane et al., 2011; Leida et al., 2012; De la Fuente et al., 2015), pear (Saito et al., 2015) and apple (Mimida et al., 2015). Progress through dormancy is first led by winter temperatures when buds accumulate chill until a threshold is met (endodormancy; Lang et al., 1987). Following endodormancy release, buds in the second phase of dormancy, namely ecodormancy, are able to resume their growth and flower under the control of warmer temperatures at the end of the winter. In the current context of climatic change, bud dormancy onset and release can be affected in a complex

way by increasing winter and spring temperatures (Menzel, 2000; Chmielewski and Rötzer, 2001), potentially leading to in lower fruit production.

Despite its importance in fruit tree production, the molecular events regulating dormancy and its control by temperature are still poorly understood. Nonetheless, genetic and genomic approaches have recently led to some progress in understanding the mechanisms underlying dormancy control in deciduous trees. In *Rosaceae*, combined studies have led to the identification of molecular mechanisms, including a cluster of *DORMANCY-ASSOCIATED MADS*-box (*DAM*) genes (Bielenberg et al., 2008). It was demonstrated in the “evergrowing (evg)” peach (*Prunus persica* (L.) Batsch) mutant that the deletions of four of six *DAM* genes are responsible for a persistent growth under low temperature and short photoperiod resulting in a non-entry in dormancy (Bielenberg et al., 2004; Bielenberg et al., 2008). A positive correlation has been found between the expression of *DAM*-related genes and the bud dormancy status, with up-regulated *DAM*-related genes in dormant buds, while they are down regulated during dormancy release and before bud break as it was especially shown for *DAM5* and *DAM6* (Jiménez et al., 2010; Yamane et al., 2011; Leida et al., 2012).

Several studies suggest that epigenetic mechanisms are implicated in the regulation of dormancy in perennial species. Recently, Rothkegel and colleagues (Rothkegel et al., 2017) showed that DNA methylations and siRNAs participate to the silencing expression of *PavMADS1* (*PpeDAM5* peach genes) during cold accumulation and dormancy release in sweet cherry. Additionally, the regulation of *DAM* genes silencing during the transition from dormancy to dormancy release in peach is under the control of the repressive histone mark H3K27me3 (De la Fuente et al., 2015). Conversely, the positive regulation of *DAM6* in peach dormant buds is led by the presence of H3K4me3 and H3ac modifications around the translation start region (Leida et al., 2012), similarly to results for *DAM1* in leafy spurge (Horvath et al., 2010).

In this study, we explore the regulation of the expression for the two key genes *PavDAM5* and *PavDAM6* in the sweet cherry cultivar ‘Burlat’ during dormancy and dormancy release using RNA-seq and ChIP-seq approaches. By coupling the ChIP-seq results for H3K4me3 and the transcriptomic analysis on the same starting material, we show a significant enrichment differences between these two dormancy key genes, correlated with variations in expression.

MATERIAL AND METHODS

Plant material:

Sweet cherry trees (cultivar ‘Burlat’) were grown in an orchard located at the Fruit Experimental Unit of INRA in Toulence (France, 44°34’N 0°16’W) under standard agricultural practices.

Flower buds used for the RNA-seq and ChIP-seq experiment were collected on 21st October 2014 and 5th December 2014 for dormant buds and 21st January 2015 for non-dormant buds. Buds for three biological replicates were pooled from 3 different branches and were flash frozen in liquid nitrogen and stored at -80°C prior performing ChIP-seq and RNA-seq.

Measurements of bud break and estimation of the dormancy release date:

Measurements for the dormancy stages were performed on cut branches from ‘Burlat’ trees, randomly chosen. The branches were harvested every two weeks between November 1st 2014 and March 15th 2015 and incubated in water pots placed in a growth chamber under optimal growth conditions (25°C, 16h light/ 8h dark, 60-70% humidity). The water was replaced every 3-4 days. After 10 days of incubation, phenological observations were carried out on flower buds. The date of dormancy release was evaluated when the percentage of bud break reached 50% at BBCH stage 53 (Meier, 2001).

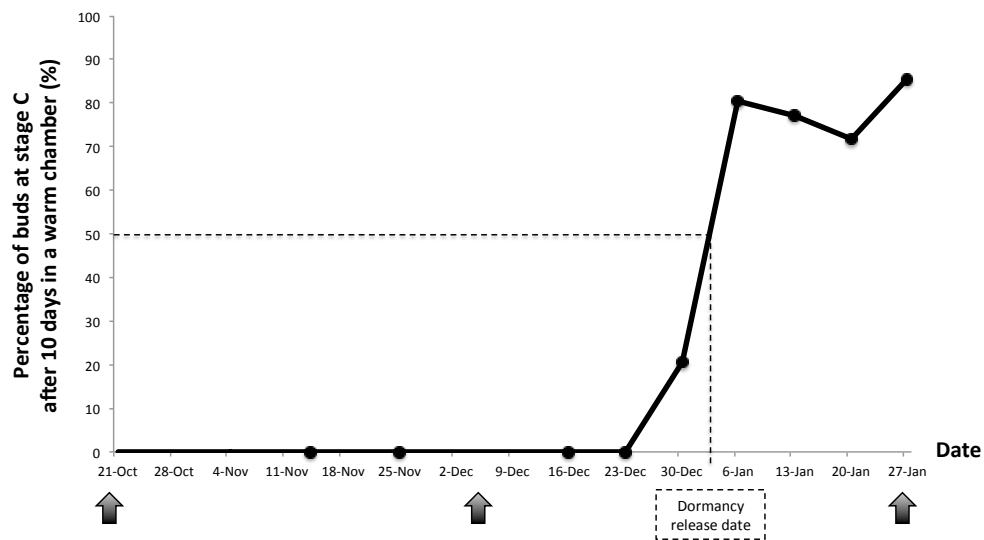


Figure 1| Measurements of bud break and estimation of the dormancy release date in 'Burlat' flower buds during the winter 2014/2015

Branches from 'Burlat' trees were collected from November 2014 to January 2015 (14th November 2014, 25th November 2014, 16th December 2014, 23th December 2014, 6th January 2015, 13th January 2015, 20th January 2015 and 27th January 2015) and kept in water pot during 10 days in growth chamber (25°C, 60-70% humidity, long days). After 10 days, the number of flower buds at BBCH stage 53 was recorded. The dotted line represents the dormancy release date when buds reach 50% bud break after 10 days in a warm chamber. The black arrows correspond to the RNA-seq and ChIP-seq time points (21th October 2014, 5th December 2014 for dormant buds and 21th January 2015 for non-dormant buds).

RNA-seq:

- RNA extraction and library preparation

Total RNA was extracted from 50 mg of frozen flower buds using RNeasy Plant Mini kit (Qiagen) with minor modifications (1.5% PVP-40 added in the RLT buffer). RNA quality was evaluated using Tapestation 4200 (Agilent Genomics). Only samples with RINe (RNA integrity number equivalent) superior or equivalent to 8.5 were used for RNA-seq. Library preparation was performed with 1 µg of total RNA using the TruSeq Stranded mRNA Library Prep Kit High Throughput (96 samples, 96 indexes, Illumina cat. no. RS-122-2103). DNA quality from libraries was evaluated using Tapestation 4200 (Agilent Genomics).

The libraries were sequenced on a NextSeq500 using paired-end sequencing of 75 bp in length.

- Data analysis

The raw reads obtained from the sequencing were analysed using several publicly available software and in-house scripts. We first determined the quality of reads using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Possible adaptor contamination and low quality trailing sequences were removed using Trimmomatic (Bolger *et al.*, 2014), before aligned to the *Prunus persica* reference genome using Tophat (Trapnell *et al.*, 2009). Possible optical duplicates resulted from library preparation were removed using the Picard tools (<https://github.com/broadinstitute/picard>). For each gene, raw reads and TPM (Transcripts Per Million) were computed (Wagner *et al.*, 2012). Data were filtered by removing lowly expressed genes and genes that have a little change in expression (coefficient of variation < 0.3) are removed. The heat map diagram was generated using z-score data and heatmap.2 function on R.

ChIP-seq :

- ChIP and library preparation

ChIP was carried out using the procedure described in Leida *et al.*, (2012) with minor modifications following:

- We optimised this protocol to start from 300 to 500 mg of buds with scales

- The cross-linking step is performed on frozen, pulverised material to study fast responses by flash freezing material immediately after harvest

- DNA extraction and purification is carried out using SPRI beads (Solid Phase Reversible Immobilisation)

Library preparation was performed using all the volume for IPs (50 μ l) and 20 ng in 50 μ l using the TruSeq ChIP Library Preparation Kit (48 samples, 12 indexes, Illumina, cat. no. IP-202-1012). DNA quality from libraries was evaluated using TapeStation 4200 (Agilent Genomics).

The libraries were sequenced on a NextSeq500 using paired-end sequencing of 75 bp in length.

o Data analysis

Sequenced ChIP-seq data were analysed in house, following the same quality control and pre-processing as in RNA-seq. The adaptor-trimmed reads were mapped to the *Prunus persica* reference genome using Bowtie2 (Langmead *et al.*, 2009). Possible optical duplicates were removed using Picard, as described earlier. Data are represented using the Integrative Genome Viewer (IGV; Robinson *et al.*, 2011) as a tool for visualising sequencing read profiles.

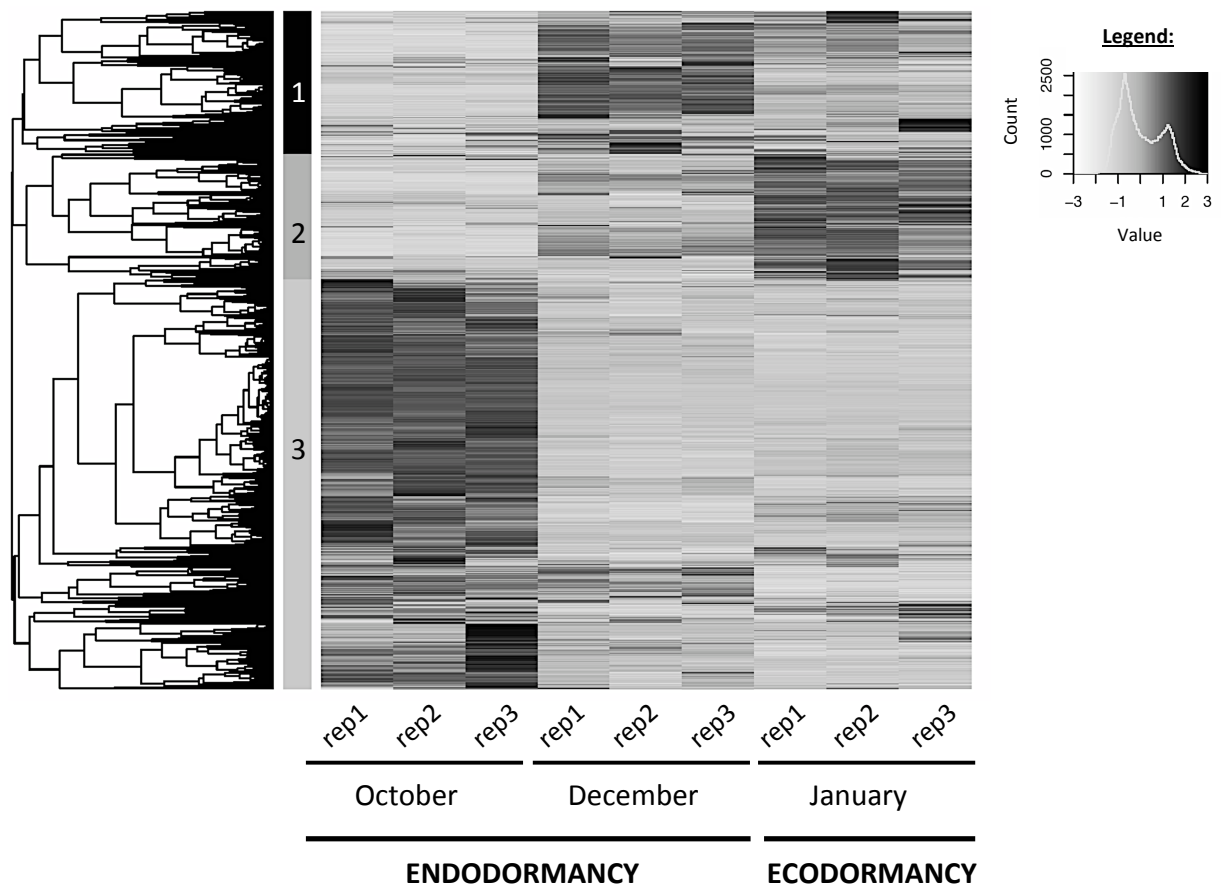


Figure 2| Heat map diagram of relative gene expressions in October, December and January in 'Burlat' flower buds

The heat map diagram was carried out using z-score for three different time points (October, December and January) and three replicates for each time point. As represented in the legend, the white/grey colours represents a relative decrease in gene expression and the black colour indicates a relative increase in gene expression.

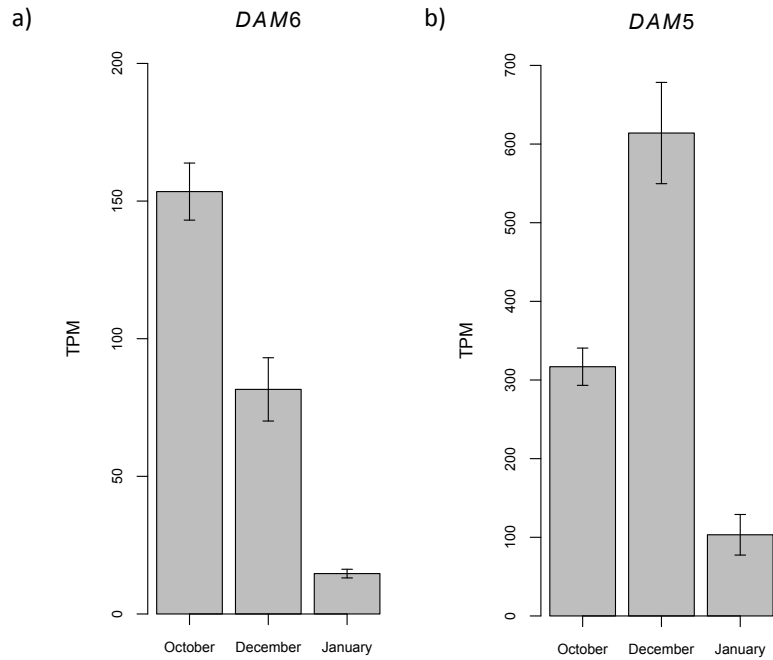


Figure 3| Relative expression of a) *DAM6* and b) *DAM5* in October, December and January in 'Burlat' flower buds

DAM6 gene is found in the cluster 3 and *DAM5* gene in the cluster 1 (Figure 2).

The expression is represented in TPM (Transcripts Per kilobase Million). Error bars indicate the range of variation between the three biological replicates. The dotted line corresponds to the dormancy release phase.

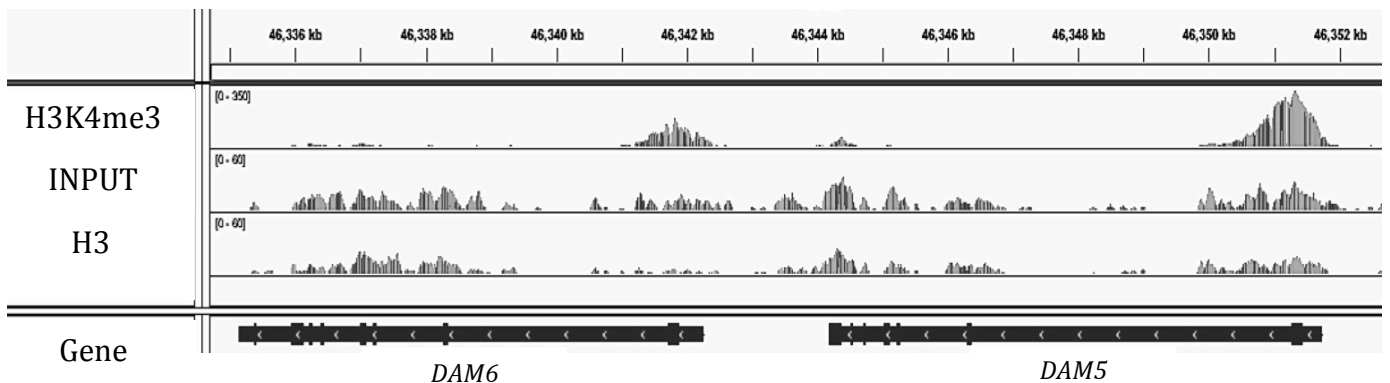


Figure 4| H3K4me3 ChIP-seq profiles in 'Burlat' flower buds in December

IGV (Robinson *et al.*, 2011) screenshot of ChIP-seq data for H3K4me3 performed on 'Burlat' flower buds in December. The upper panel shows the H3K4me3 enrichment along *DAM6* and *DAM5* genes. INPUT and H3 enrichment are present as controls showing that the enrichment peaks found in the first exon of *DAM6* and *DAM5* genes are biologically correct. Genes are represented by black rectangles, with white arrows indicating gene directionality and taller boxes within the rectangles representing exons.

RESULTS and DISCUSSION

Dormancy phase identification of cherry flower buds: endodormancy and ecodormancy

The two main physiological and developmental phases implicated in perennial dormancy (Lang *et al.*, 1987) were identified in the 'Burlat' cultivar. The endodormancy phase started around

October and was maintained until 23th December 2014 while and the ecodormancy phase lasted from 30th December to late January 2015. The dormancy release date was estimated on 2nd January 2015 (Figure1).

Endodormancy and ecodormancy consists of a positive succession of key genes expression

A transcriptomic analysis was carried out to gain an understanding of how the transcriptome changes during the dormancy cycle. The RNA-seq analysis led to the identification of three main cluster of gene expression patterns corresponding to groups of genes upregulated at the three different dates (Figure 2). The cluster containing the highest number of genes corresponds to genes highly expressed at the beginning of endodormancy (October) followed by a downregulation at the end of endodormancy and ecodormancy (cluster 3, Figure 2). Similar trend is found for the other two dormancy phases: cluster 1 includes genes upregulated only at the end of endodormancy (December) while cluster 2 covers genes expressed during the ecodormancy phase (January; Figure 2). Therefore, results suggest that physiological and developmental dormancy phases can be categorized as a series of timely up- and down-regulated genes.

PavDAM6 and PavDAM5 expression level is associated with chromatin modifications

Mapping results from the transcriptomic data allowed the identification of *Prunus avium* key genes *PavDAM5* and *PavDAM6*, corresponding to the peach *PpeDAM5* and *PpeDAM6*. We further analysed the expression of *PavDAM6* and found that it was part of cluster 3, being highly expressed in October, at the beginning of dormancy and then down-regulated over time and cold exposure (Figure 3a). Conversely, *PavDAM5* was found to be highly expressed at the end of endodormancy (cluster 1; Figure 3b). Both *PavDAM5* and *PavDAM6* genes were down-regulated after dormancy release, suggesting their role in the maintenance of endodormancy, similarly to results for peach (Bielenberg et al., 2008; Yamane et al., 2011; De la Fuente et al., 2015), japanese apricot (Sasaki et al., 2011; Saito et al., 2014), pear, chinese cherry (Zhu et al., 2015).

The tri-methylation of histone H3 at lysine 4 (H3K4me3) is associated with open chromatin and gene activation (Santos-Rosa et al., 2002). We conducted a ChIP-seq against the H3K4me3 histone mark in order to correlate the abundance of histone marks to the expression patterns of *PavDAM6* and *PavDAM5* for the same time point (5th December 2014), corresponding to endodormancy. We found a H3K4me3 enrichment around the translation start of both *PavDAM6* and *PavDAM5* during dormancy (Figure 4). This result is consistent with the results of Leida et al. (2012) where an enrichment of this histone mark was found around the translation start of *PpeDAM6*. And similarly to results in leafy spurge (Horvath et al., 2010) where the expression peak of *DAM1* is correlated with the presence of H3K4me3 at the translation start of this gene. H3K4me3 abundance on *PavDAM5* locus is higher than the abundance on *PavDAM6* locus, which can be correlated with the difference in their expression levels (Figure 3). Indeed, the *PavDAM5* expression peak is five-times higher than the *PavDAM6* expression peak at the end of endodormancy (Figure 3). Therefore, the difference of trimethylation abundance of H3K4 might play a role in the regulation of dormancy

CONCLUSION

In this study, dormancy in *Prunus avium* L. appeared to be regulated by the succession of *DAM* genes up-regulation and repression, *PavDAM6* being first up-regulated at the beginning of endodormancy then down-regulated over dormancy. This expression pattern is followed in time by an increase in *PavDAM5* expression, with a potential role for bud dormancy maintenance. These results prove the implication of *PavDAM6* and *PavDAM5* in the regulation of dormancy in sweet cherry. Additionally, we showed the positive correlation between the abundance of

H3K4me3 at *PavDAM6* and *PavDAM5* loci and the expression level of the two genes. The abundance of H3K4m3 may play an important role in the dormancy and dormancy release regulation. However, more time points are required to better follow the dynamics of H3K4me3 during the dormancy establishment and release.

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Hormonal control of dormancy in sweet cherry flower buds

Noémie Vimont^{1,2,3}, Rémi Beauvieux¹, Adrian Schwarzenberg², Philip Wigge³, Elisabeth Dirlewanger¹, Bénédicte Wenden¹

¹INRA, UMR Biologie du Fruit et Pathologie, Univ. Bordeaux, Villenave d'Ornon, France; ²Agro Innovation International, CMI, Saint-Malo, France; ³The Sainsbury Laboratory, University of Cambridge, Cambridge, United Kingdom

Abstract

In the context of global change, temperate fruit trees are affected by contradictory effects: increased productivity in relation with longer growing season and insufficient chilling during winter. It is therefore essential to better understand the mechanisms controlling phenology and its response to environmental conditions. Optimal timing and quality of flowering directly depend on adequate dormancy progression during winter and spring, regulated by a combination of chilling and warm temperatures. Physiological, genetic and functional genomic studies have shed light onto the mechanisms underlying dormancy control in deciduous trees. Notably, internal signals such as hormones and sugars were shown to play a key role in dormancy establishment, maintenance and release. In order to further study how these signaling pathway control dormancy progression, we combined transcriptional analyses and quantification of abscisic acid (ABA) and gibberellins (GAs) in sweet cherry flower buds during the dormancy period. Our results suggest that ABA is critical for dormancy maintenance and we propose that the complex balance between ABA and GA pathways regulate the timing for dormancy release.

Keywords: *Prunus avium* L., bud dormancy, phenology, abscisic acid, gibberellins

INTRODUCTION

Perennial plants have evolved strategies that enhance survival under the various environmental stresses they face during their growth and reproductive cycles. Among them, dormancy is a quiescent phase that protects meristematic and reproductive tissues from freezing damage. In temperate trees, buds enter winter dormancy under the control of decreasing photoperiod and temperatures (Heide and Prestrud, 2005; Singh et al., 2016). Subsequently, endodormancy, when buds are unable to resume growth even under favorable conditions (Rohde and Bhalerao, 2007), is followed by ecodormancy, when bud development is inhibited by unfavorable conditions until warm spring temperatures trigger bud break and flowering (Lang et al., 1987). In the current context of climate change, temperate fruit trees are affected by contradictory effect: longer growing season associated with advanced flowering time under increasing temperatures, and insufficient chilling induced by mild winter temperatures, both dramatically impacting growth and production (Atkinson et al., 2013; Vitasse et al., 2011). It is therefore essential to better understand the mechanisms controlling dormancy and the response to environment in order to anticipate temperate fruit tree adaptation to future climatic conditions. Dormancy control by temperature has been a focus for decades and physiological, genetic and functional genomic studies have shed some light onto the mechanisms involved in perennial plants (Beauvieux et al., 2018; Cooke et al., 2012; Ríos et al., 2014). These studies have shown that bud dormancy is controlled by a complex array of pathways that integrate endogenous and environmental signals. In particular, the major role of hormones in the regulation of bud growth cessation, dormancy and activity resumption has been extensively discussed (Beauvieux et al., 2018; Cooke et al., 2012), including the common features that bud and seed dormancy share: gibberellins (GA) promote growth whereas abscisic acid (ABA) promotes dormancy (Leida et al., 2012; Tuan et al., 2018; Wang et al., 2016).

ABA plays a central role in the regulation of dormancy onset and maintenance through the repression of bud activity. Under autumn short days and decreasing temperatures, ABA is involved in dormancy onset (Li et al., 2018; Rohde and Bhalerao, 2007; Tuan et al., 2017;

Tylewicz et al., 2018) and dormancy maintenance (Chmielewski et al., 2017; Li et al., 2018; Wang et al., 2016; Wen et al., 2016; Zhang et al., 2018; Zheng et al., 2015). Recent transcriptomic analyses have further investigated the genes involved in the balance between ABA biosynthesis, including key genes 9-cis epoxy-carotenoid dioxygenases (*NCED*), and catabolism, mainly controlled by *CYP707A*, encoding cytochrome P450 monooxygenases that inactivate ABA into 8'-hydroxy ABA (Bai et al., 2013; Khalil-Ur-Rehman et al., 2017; Li et al., 2018; Wang et al., 2016; Zhang et al., 2018; Zheng et al., 2018; Zhong et al., 2013; Zhu et al., 2015). Similarly, recent reports have revealed that GAs play a major role in maintaining growth before the induction of dormancy (Eriksson et al., 2015; Ruttink et al., 2007; Singh et al., 2016) and promoting growth during ecodormancy (Wen et al., 2016; Zhang et al., 2018). Although transcriptomic results for GA 2-oxidases (*GA2ox*), GA 3-oxidases (*GA3ox*), GA 20-oxidases (*GA20ox*) vary between studies and therefore suggest complex and distinct functions, studies show that GA biosynthesis genes *GA20ox* and *GA3ox* decrease during dormancy induction then increases after dormancy release while GA deactivation *GA2ox* genes are up-regulated during endodormancy and inhibited after endodormancy is released (Bai et al., 2013; Khalil-Ur-Rehman et al., 2017; Wen et al., 2016; Yamaguchi, 2008; Zhang et al., 2018; Zheng et al., 2018; Zhu et al., 2015).

In this study, we investigated ABA and GA roles in sweet cherry flower bud dormancy. We monitored endogenous ABA and GA contents, together with the expression of genes related to ABA and GA metabolism throughout flower bud dormancy in the sweet cherry cultivar 'Regina'.

MATERIAL AND METHODS

Plant material

Samples were collected from sweet cherry (*Prunus avium* L.) 'Regina' trees grown in an orchard located at the Fruit Experimental Unit of INRA (Bourran, France, 44° 19' 56" N, 0° 24' 47" E) under standard agricultural practices. Flower buds were sampled on-site from two trees between July 2015 and March 2016. Upon harvested, buds were flash frozen in liquid nitrogen and stored at -80°C.

In addition, every two weeks, three branches were cut and incubated in water in a growth chamber under forcing conditions (25°C, 16h light/8h dark, 60% humidity). After ten days, flower bud break was evaluated for each bud, corresponding to BBCH stage 53 (Fadón et al., 2015). Dormancy release date was recorded as the date when 50% of the flower buds reached the BBCH stage 53 after ten days under forcing conditions.

Phytohormone extraction

For each sample, 10 mg of frozen pulverised flower buds were weighed in a 2 mL tube. The extraction was carried out by adding 1 mL of cold 70% MeOH / 29% H₂O / 1.0% formic acid, containing isotopically labelled internal standards. Then, the tubes were stirred at room temperature for 30 min and centrifuged (5427R, Eppendorf) at 16,000 rpm for 20 minutes at 4°C. The supernatant of each tubes were transferred into new tubes and evaporated to dryness using a Turbovap LV system (Biotage, Sweden). The dried extracts were dissolved with 1 mL of a 2% formic acid solution. The resuspended extracts were purified using a solid phase extraction (SPE) Evolute express ABN 1ml-30 mg (Biotage, UK). The eluate was evaporated to dryness and resuspended in 200 µL of 0.1% formic acid.

Phytohormones quantification

ABA and GA₄ were quantified by UHPLC-MS/MS. ABA, GA₄, [²H₆]-ABA, and [²H₂]-GA₄ were purchased from OlchemIn (Olomouc, Czech Republic). Phytohormones were analyzed by an UHPLC-MS/MS system. The separation and detection were achieved using a Nexera X2 UHPLC system (Shimadzu, Japan) coupled to a QTrap 6500+ mass spectrometer (Sciex, Canada) equipped with an electrospray (ESI) source. Phytohormones separation was carried out by injecting 2 µL into a Kinetex Evo C18 core-shell column (100 x 2.1mm, 2.6µm, Phenomenex,

USA) at a flow rate of 0.7 mL/min, and the column oven was maintained at 40°C. The mobile phases were composed of solvent A Milli-Q water (18 M Ω , Millipore, USA) containing 0.1% formic acid (LCMS grade, Fluka analytics, Germany), and solvent B acetonitrile LCMS grade (Fisher Optima, UK) containing 0.1% formic acid. The gradient elution started with 1% B, 0.0-5.0 min 60% B, 5.0-5.5 min 100% B, 5.5-7.0 min 100% B, 7.0-7.5 min 1% B, and 7.5-9.5 min 1% B. The ionization voltage was set to 5kV for positive mode and -4.5 kV for negative mode producing mainly [M+H]⁺ and [M-H]⁻ respectively. The analysis was performed in scheduled multiple reaction monitoring (MRM) mode in positive and negative mode simultaneously with a polarity switching of 5 ms. All quantitative data were processed using MultiQuant software V 3.0.2 (Sciex, Canada).

RNA extraction and library preparation

Total RNA was extracted from 50-60 mg of frozen and pulverised flower buds using RNeasy Plant Mini kit (Qiagen) with minor modification (1.5% PVP-40 was added in the RLT buffer). RNA quality was evaluated using Tapestation 4200 (Agilent Genomics). Only samples with RNA integrity number equivalent (RINe) superior or equivalent to 8.5 were used for RNA-seq. Library preparation was performed with 1 μ g of total RNA using the TruSeq Stranded mRNA Library Prep Kit High Throughput (96 samples, 96 indexes, Illumina cat. no. RS-122-2103). DNA quality from libraries was evaluated using Tapestation 4200 (Agilent Genomics). The libraries were sequenced on a NextSeq500 using paired-end sequencing of 75 bp in length.

RNA-seq data analysis

The raw reads obtained from the sequencing were analysed using several publicly available software and in-house scripts. The quality of reads was assessed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and possible adaptor contaminations and low quality trailing sequences were removed using Trimmomatic (Bolger et al., 2014). Trimmed reads were mapped to the peach (*Prunus persica* (L.) Batsch) reference genome v2.0 (Verde et al., 2013) using Tophat (Trapnell et al., 2009) and possible optical duplicates were removed using Picard tools (<https://github.com/broadinstitute/picard>). For each gene, Transcripts Per Million (TPM) were calculated (Wagner et al., 2012).

RESULTS AND DISCUSSION

Dormancy depth is correlated with endogenous ABA content

Several studies have highlighted a strong correlation between ABA content and dormancy status. To address this issue in sweet cherry flower buds, we measured ABA levels during the bud development cycle, from organogenesis in July to flowering in the spring in the late flowering sweet cherry cultivar 'Regina' and results confirmed an increase in ABA content during the early stages of dormancy in September and October (Figure 1). In addition, highest ABA levels were synchronized with endodormancy (November, December), followed by a marked decrease prior to dormancy release, recorded on February 26th.

To further investigate the ABA pathway regulation during dormancy, we examined sweet cherry gene predictions for genes involved in ABA biosynthesis (*PavNCED*) and catabolism (*PavCYP707*). Expression for *PavNCED1* and *PavNCED4* genes was not correlated with ABA levels while expression patterns for *NCED3* and *PavNCED5* genes seemed strongly linked to ABA contents and dormancy status (Figure 2), thus suggesting their key role in the regulation of ABA levels during dormancy. In particular, *PavNCED5* expression showed a sharp increase during dormancy onset and a marked decay before dormancy release. An increase in *PavNCED3* expression occurred when *PavNCED5* expression decreased. On the other hand, ABA 8'-hydroxylases (*CYP707A* genes) are considered the major regulatory enzymes for ABA catabolism, and indeed, low ABA levels were associated with increased expression for *PavCYP707A4* after dormancy release (Figure 2). By contrast, *PavCYP707A2* is characterized by a sharp increase during endodormancy, more specifically in December, but it is not related to a

visible decrease in ABA content. In addition, we identified *PavUGT71B6*, a sweet cherry ortholog of the *Arabidopsis thaliana* *UDP-glycosyltransferase 71B6*, that preferentially glycosylates ABA into ABA-GE (Priest et al., 2006), and is up-regulated concomitantly with ABA levels, suggesting that ABA homeostasis is regulated through esterification when ABA content is high. Expression for genes involved in ABA signaling, including receptor *PavPYL8* and response *PavABF2* and *PavABF3* genes was activated during dormancy, consistently with high ABA levels (Figures 1 and 2).

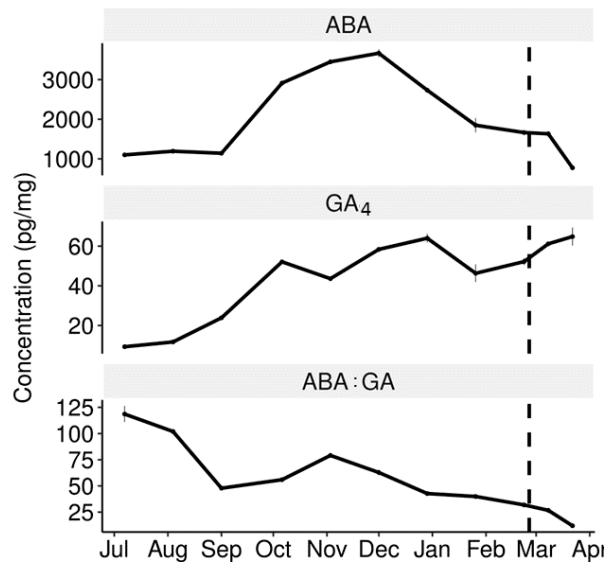


Figure 1. Levels of endogenous active ABA and GA₄, and ABA:GA ratio in the flower buds of sweet cherry cultivar ‘Regina’ during bud development. Error bars indicate the data range between the biological replicates. Dotted lines represent dormancy release. ABA: Abscisic acid; GA: Gibberellic acid.

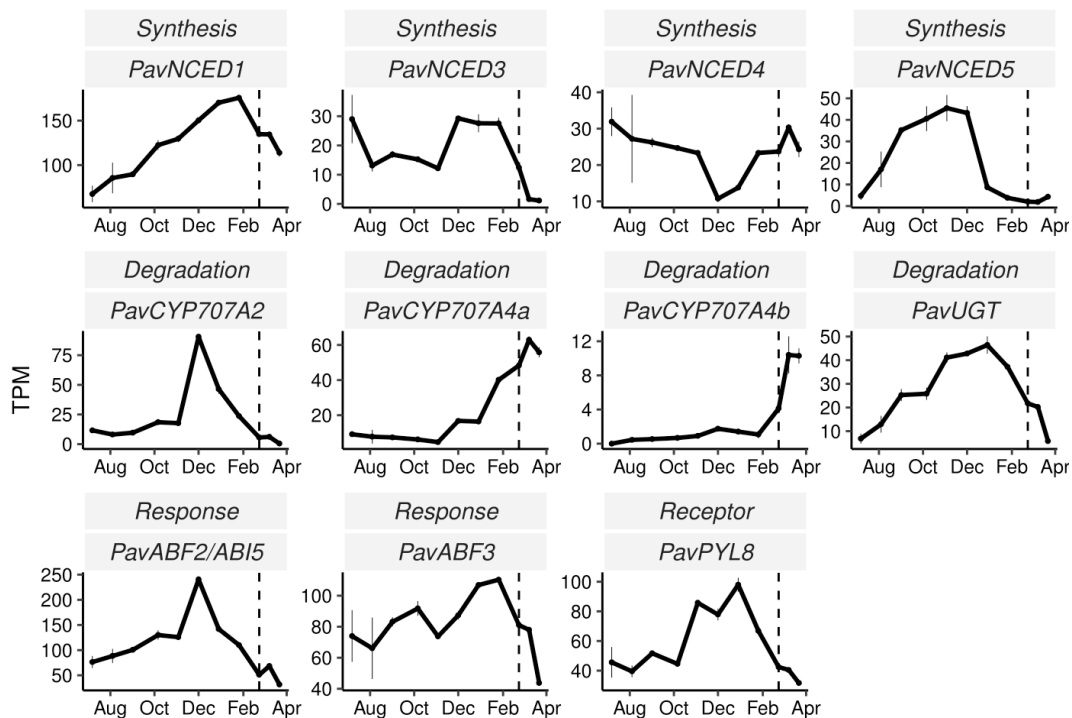


Figure 2. Transcriptional dynamics of genes associated with ABA pathway in the flower buds of sweet cherry cultivar ‘Regina’ during bud development. Expression of specific genes involved in ABA biosynthesis

pathway, degradation, signal transduction and response are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the data range between the biological replicates. Dotted lines represent dormancy release. NCED: 9-cis epoxy-carotenoid dioxygenases; CYP: cytochrome P450 monooxygenases; UGT: UDP-glycosyl transferase; ABF2/3: ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2/3; ABI5: ABSCISIC ACID INSENSITIVE 5; PYL8: PYRABACTIN RESISTANT LIKE 8.

GA metabolism increases during flower bud dormancy progression

In recent studies, distinct functions were identified for gibberellins during bud dormancy (Zheng et al., 2018; Zhuang et al., 2013). To test whether these results could be confirmed in sweet cherry buds, bioactive GA₄ levels were measured during the bud development cycle in the late flowering sweet cherry cultivar 'Regina' (Figure 1). Interestingly, GA₄ levels increased from September, when photoperiod and temperatures decrease, onward, with high levels during dormancy, as opposed to low GA levels recorded in grapevine and Japanese apricot buds during dormancy maintenance (Zhang et al., 2018; Zheng et al., 2018). These observations also differ from results obtained in grapevine with high GA₁ levels during dormancy induction (Zheng, et al., 2018) or in Japanese apricot, characterized by high levels of GA₃, especially during ecodormancy (Zhang et al., 2018). Enhanced levels of GA₄ were synchronized with expression peaks of GA biosynthesis genes *PavGA20ox2* and *PavGA3ox* (Figure 3). The last step of active GA biosynthesis relies on the activity of GA3ox essentially for the production of GA₁ and GA₄. *PavGA3ox1a* was the only *GA3ox* transcript detected during dormancy (Figure 3) and its expression sharply increases during the early stages of dormancy in October and reaches its highest expression value at maximum dormancy depth in December, and is then downregulated after dormancy release. We identified three *PavGA2ox*, potentially involved in GA inactivation (Figure 3). *PavGA2ox8b* was highly expressed during endodormancy, concomitantly with *PavGA3ox1a* expression, thus suggesting a balance between synthesis and degradation that closely controls the levels of bioactive GAs. *PavGA2ox8c* and *PavGA2oxb* were expressed before dormancy and during the early stages of dormancy.

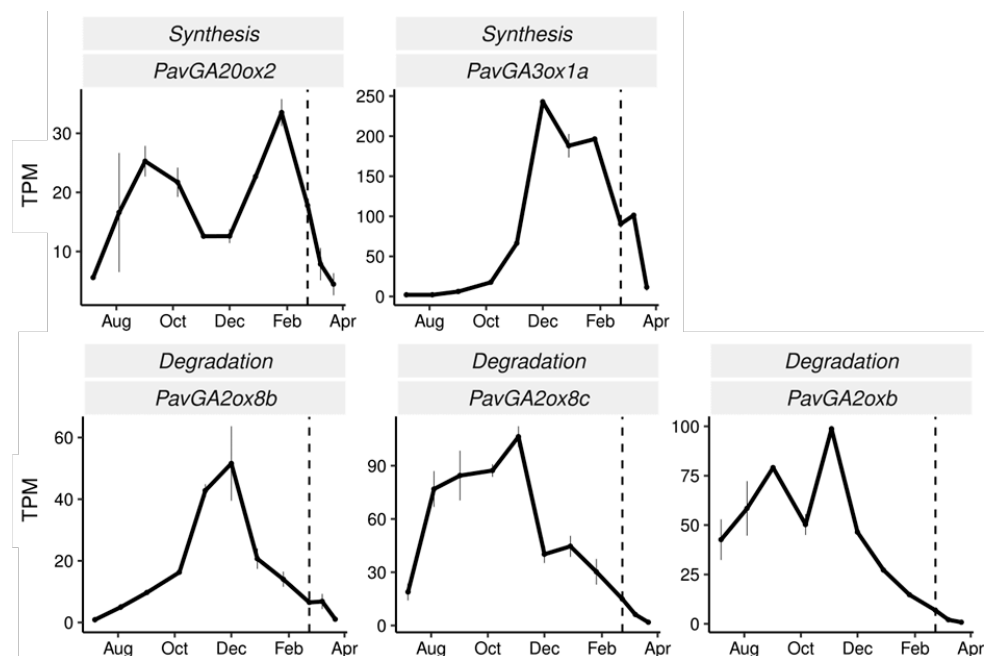


Figure 3. Transcriptional dynamics of genes associated with GA pathway in the flower buds of sweet cherry cultivar 'Regina' during bud development. Expression of specific genes involved in ABA biosynthesis pathway, degradation, signal transduction and response are represented in TPM (Transcripts Per kilobase Million). Error bars indicate the data range between the biological replicates. Dotted lines represent dormancy release. GA20ox: GA 20-oxidase; GA2ox: GA 2-oxidase; GA3ox: GA 3-oxidase.

A complex balance between ABA and GA may regulate the timing of bud dormancy release

ABA and GA balance was shown to integrate internal and external cues to control plant growth in seed dormancy (Rodríguez-Gacio et al., 2009; Tuan et al., 2018) and Zhang and colleagues (2018) recently confirmed that ABA/GA₃ ratio markedly decreased around dormancy release in Japanese apricot. In 'Regina' flower buds, ABA/GA₄ was very high during organogenesis and decreased until September, related to increasing GA₄ content prior to dormancy onset (Figure 1). ABA/GA₄ ratio increased again during dormancy onset and was at the highest value in November, corresponding to deep endodormancy. Following this stage, the ABA/GA₄ ratio steadily decreased until dormancy release (Figure 1). According to these observations, we propose that a threshold for ABA/GA₄ determines when dormancy release is triggered, namely 30 for our results (Figure 1). Therefore, the ABA/GA₄ balance finely controls the bud dormancy-growth resumption cycle in sweet cherry flower buds. ABA and GA signaling pathways are closely regulated by biosynthesis and catabolism mechanisms involving genes with distinct functions during dormancy progression.

CONCLUSION

In this study, we investigated the role of GA and ABA balance in sweet cherry flower bud dormancy progression. We showed that ABA and GA₄ levels are high during endodormancy and that the balance between the two hormone signaling pathways may regulate the timing of dormancy release. Genes involved in ABA and GA biosynthesis and catabolism are therefore crucial factors for the control of bud dormancy by mediating the ABA/GA₄ balance. Similar findings in seed dormancy have led to the development of mathematical models for seed germination based on the endogenous hormone integration system (Topham et al., 2017). Accordingly, ABA/GA balance could be integrated into phenology models, thus opening new roads for perennial studies.

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