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Etude de la régulation transcriptionnelle et post-transcriptionnelle médiées par la température de la dormance et du débourrement chez le pommier.

Présentée par Julio GARIGHAN

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Sous la direction de Evelyne COSTES et Fernando ANDRES-LALAGUNA

Devant le jury composé de

Isabel ALLONA, Professeure, Universidad Politécnica de Madrid	Rapporteure
Catherine BELLINI, Directrice de Recherche, INRAE/UPSC	Rapporteure
Laurent TORREGROSSA, Professeur, Institut Agro Montpellier SupAgro	Examinateur
Jean-Marc CELTON, Professeur associé, Université d'Angers	Examinateur
Luis REVES, Directeur de Recherche, EMBRAPA	Examinateur, Invité
Evelyne Costes, Directrice de Recherche, INRAE	Directrice de thèse
Fernando Andres, Chargé de Recherche, INRAE	Co-encadrant de thèse

UNIVERSITÉ DE MONTPELLIER

Montpellier SupAgro

ABSTRACT

Temperate and boreal trees face harsh conditions during winters such as extreme cold, drought and insufficient light. These trees have developed a specific mechanism called “bud dormancy” to protect their meristem and floral tissues during winter and allow the next growth cycle to occur at an appropriate time in the next spring. Three successive stages are usually considered during dormancy. In this thesis, I focused on two of them, endo- and eco-dormancy, and the transition between them. In the endo-dormant stage, bud growth is not possible even when placed under forcing conditions (i.e., warm temperatures). This stage requires exposure to cold temperatures to be overcome. Then, the buds enter the ecodormancy stage, in which unsuitable environmental conditions repress growth. Once in warmer conditions, the tree opens its buds (bud break) and enters into the development and reproductive phase.

In this Ph.D., I have studied dormancy in the apple tree (*Malus domestica* Borkh), an economically important fruit crop that presents chilling-dependent bud dormancy. The chilling requirements (CR) necessary to overcome endodormancy are known to vary among cultivars. CR is thus genetically defined, and genes such as *DORMANCY ASSOCIATED MADS-box* genes (*DAMs*) have been reported as main dormancy regulators. In *A. thaliana,* MADS-box genes act in the vernalization and the thermosensory pathways to control flowering time in response to ambient temperature. These pathways are partly modulated at the transcriptional and post-transcriptional levels. At the post-transcriptional level, alternative splicing (AS) mechanisms and microRNAs (miRNAs) are known to operate in the temperature-mediated control of flowering in *Arabidopsis thaliana*. In this Thesis, my working hypothesis is that the temperature-mediated regulation of dormancy and bud break in apple (and other fruit trees) involve similar mechanisms to those that operate in the vernalization and thermosensory flowering pathways of *A. thaliana*. Thus, I investigated differentially expressed genes and miRNAs during endormancy and transition to ecodormancy. I performed a preliminary analysis to detect alternative splicing (AS) events to understand better the post-transcriptional regulation of dormancy in the apple tree.

Using a transcriptomic approach, I performed RNA and small RNA sequencing analysis to study the differential expression of genes and miRNAs during

endodormancy release and transition to ecodormancy. This highlighted a huge reprograming in gene expression during this dormancy switch, with a high number of genes being up-regulated during endodormancy release. We detected two isoforms of AS for the dormancy-related *Malus domestica* (*Md*) gene *SHORT VEGETATIVE PHASE a* (*MdSVPa*) with potentially different functions. Moreover, the expression of some of the dormancy-associated genes were sensitive to temperature variation and/or Reactive Oxidative Species (ROS). Furthermore, two cultivars of apple trees with different chilling requirements presented other gene expressions, especially in the apple homologs of the *A. thaliana HOMEOBOX PROTEIN 22* (*HB22*) and *AINTEGUMENTA* (*ANT*) genes. We could identify 17 differentially expressed during endodormancy release among the detected miRNAs. In particular, the expression pattern of miR159a suggests a role of this miRNA during dormancy linked to the abscisic acid perception.

This work brings new knowledge on the role of AS and miRNAs in bud dormancy post-transcriptional regulation, even though more studies are necessary to fully understand their impact in such complex mechanisms as bud dormancy.

RÉSUMÉ

Les arbres des régions tempérées et boréales font face à de rudes conditions environnementales au cours de l'hiver, telles que des températures froides extrêmes, la sécheresse et le manque de lumière. Ces arbres ont développé un mécanisme spécifique appelé "dormance" pour protéger leurs méristèmes et tissus floraux pendant l'hiver et permettre l'ouverture des bourgeons au moment approprié au printemps suivant. Trois stades de dormance sont généralement considérés. Dans cette thèse, je me suis focalisé sur deux d'entre eux, l'endo- et l'éco-dormance, et sur la transition de l'un à l'autre. Pendant l'endo-dormance, la croissance des bourgeons n'est pas possible même quand ils sont mis en conditions de forçage (i.e. températures chaudes). Ce stade requiert une exposition prolongée au froid pour être dépassé. Les bourgeons entrent alors en éco-dormance, stade au cours duquel la croissance reste réprimée tant que les conditions ne sont pas favorables. Quand les conditions sont plus chaudes, l'arbre ouvre ses bourgeons et entre en phase de croissance et de reproduction.

Dans cette thèse, j'ai étudié la dormance chez le pommier (*Malus domestica* Borkh), une espèce fruitière d'importance économique et qui présente une dormance hivernale. Les quantités de froid nécessaires à la levée de l'endo-dormance varient en fonction des cultivars. Les besoins en froid (ou chilling requirements, CR) sont donc supposés génétiquement contrôlés et des membres d'une famille de gènes appelés DORMANCY ASSOCIATED MADS-box (*DAMs*) ont été identifiés comme régulateurs majeurs de la dormance.

Chez *A. thaliana,* les gènes MADS-box agissent dans la voie de la vernalisation qui conduit à la floraison printanière. Leur régulation épigénétique par des petits ARN (miRNAs) lors d'une exposition au froid a été décrite chez cette espèce. En faisant l'hypothèse de similarités entre la vernalisation et la dormance des bourgeons, j'ai étudié les gènes et les petits ARNs différentiellement exprimés durant l'endo-dormance et dans la transition vers l'éco-dormance. J'ai aussi réalisé une analyse préliminaire de l'existence d'événements d'épissage alternatif pour mieux comprendre la régulation post-transcriptionnelle des gènes impliqués dans la dormance du pommier.

Par une approche transcriptomique, j'ai analysé les ARN et petit ARN différentiellement exprimés durant l'endodormance et la transition vers l'éco-dormance. Cela a révélé une importante reprogrammation de l'expression des gènes au cours de cette transition, avec une grande quantité de gènes sur-exprimés au moment de la levée de l'endo-dormance. Deux isoformes du gène *Malus domestica* (*Md*) *SHORT VEGETATIVE PHASE a* (*MdSVPa*) ont été identifiées, dont les fonctions semblent différentes. Plusieurs des gènes associés à la dormance se sont révélés sensibles aux variations de températures et à l'application d'espèces activées de l'oxygène (ROS). De plus, deux génotypes dont les besoins en froid sont contrastés ont présenté des niveaux différents d'expression de gènes, notamment les homologues chez pommier de *A. thaliana HOMEOBOX PROTEIN 22* (*HB22*) et *AINTEGUMENTA* (*ANT*). Parmi les petits ARN, 17 se sont révélés différentiellement exprimés au cours de la levée de dormance. En particulier, le pattern d'expression du précurseur de miR159a suggère que ce petit ARN pourrait avoir un rôle, possiblement en lien avec la perception de l'acide abscissique, au cours de l'éco-dormance. Ce travail apporte de nouvelles connaissances sur le rôle des gènes impliqués dans la dormance, leur épissage alternatif et leur régulation par les petits ARN, bien que des études ultérieures soient nécessaires pour investiguer plus avant un mécanisme aussi complexe que la dormance des bourgeons.

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LIST OF ABBREVIATIONS

ABA - Abscisic Acid

ANT - AINTEGUMENTA

AP2/ERF - APETALA2/ETHYLENE RESPONSIVE FACTOR

APx - ASCORBATE PEROXIDASE

ARR -TYPE-A RESPONSE REGULATOR

AS - Alternative Splicing

BAM2 - BARELY ANY MERISTEM 2

BR - Brassinosteroids

BRR -TYPE-B RESPONSE REGULATOR

CBF - C-REPEAT BINDING FACTORS

CK - Cytokinins

COR15a - COLD-REGULATED15A

CR - Chilling Requirement

CU - Chilling Units

DAM - DORMANCY-ASSOCIATED MADS-box

DASI - Differentially Alternatively Spliced Isoform

DEG - Differentially Expressed Gene

DREB - DEHYDRATION-RESPONSIVE ELEMENT BINDING

ET - Ethylene

FDR – False Discovery Rate

FLC - FLOWERING LOCUS C

FLM - FLOWERING LOCUS M

FT - FLOWERING LOCUS T

GA - Gibberellins

GASA6 - GA-STIMULATED ARABIDOPSIS 6

GD - Golden delicious

GDDH13 – Golden delicious Double Haploid

GDV - Genome Data Viewer

GID1 - GA receptor GIBBERELLIN INSENSITIVE 1

GNR - Gene Regulatory Network

HC - Hydrogen Cyanamide

HB22 - HOMEOBOX PROTEIN 22

HR – heat Requirement

microRNAs - microRNAs

mRNA - messenger RNA

NAC - NO APICAL MERISTEM

PL - Pink Lady

QTL - Quantitative Trait Loci

RD26 - RESPONSIVE TO DESICCATION 26

RdDM - RNA-directed DNA methylation

ROS - Reactive Oxygen Species

SAM - shoot apical meristems

SOC1 - SUPPRESSOR OF OVEREXPRESSION OF CONSTANS

SPL - SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE

STZ - Salt tolerance zinc finger

SVP - SHORT VEGETATIVE PHASE

TF - Transcription Factor

TraPR - Trans-kingdom, rapid, affordable Purification of RISCs

VRN2 - VERNALIZATION 2

GENERAL INTRODUCTION

INTRODUCTION

DORMANCY AS AN ADAPTATIVE MECHANISM

Boreal and temperate trees have developed, during their evolution, adaptive mechanisms that enable their survival in extreme conditions of low temperature and lack of light. One of those mechanisms is bud dormancy, which is defined by the stop of visible growth until favorable developmental conditions are re-established [1]. Among the main morphological and physiological aspects involved in this process, we can cite the cessation of apical growth, the formation of scales and buds, leaf senescence and fall, and the acquisition of tolerance to cold and dryness [2]. The buds are vegetative structures resulting from modifications of the leaf primordia into rigid scales that protect the next leaf primordia and meristematic tissues from freezing and desiccation. In this context, they contain primary meristematic organs that will lead to the formation of shoots. They are present in most dicotyledonous plants growing under temperate conditions and guarantee that new growth and the reproductive cycle will occur in perennial species [3]. From physiological studies, bud dormancy has been divided into three phases: para-, endo- and ecodormancy [4]. Paradormancy is the growth inhibition of all meristems of a given shoot that is induced by internal signals in the plant. It is related to correlative inhibitions that include apical dominance over axillary buds and inhibition of apical growth by other parts of the plant, possibly axial tissues [5]. Endodormancy is determined by internal factors that do not allow the bud to burst even in favorable growth conditions. Finally, ecodormancy is mediated by external factors, such as cold and hydric stress that, when overcome, allow growth to resume [4]. The mechanisms involved in the dormancy regulation process are complex. They allow a fine control of tree growth, involving several aspects such as environmental stimuli, molecular regulation, hormonal signaling and epigenetic modifications. The organized set of all these factors leads to both the induction of dormancy and the resume of growth at appropriate time depending on the geographic zone.

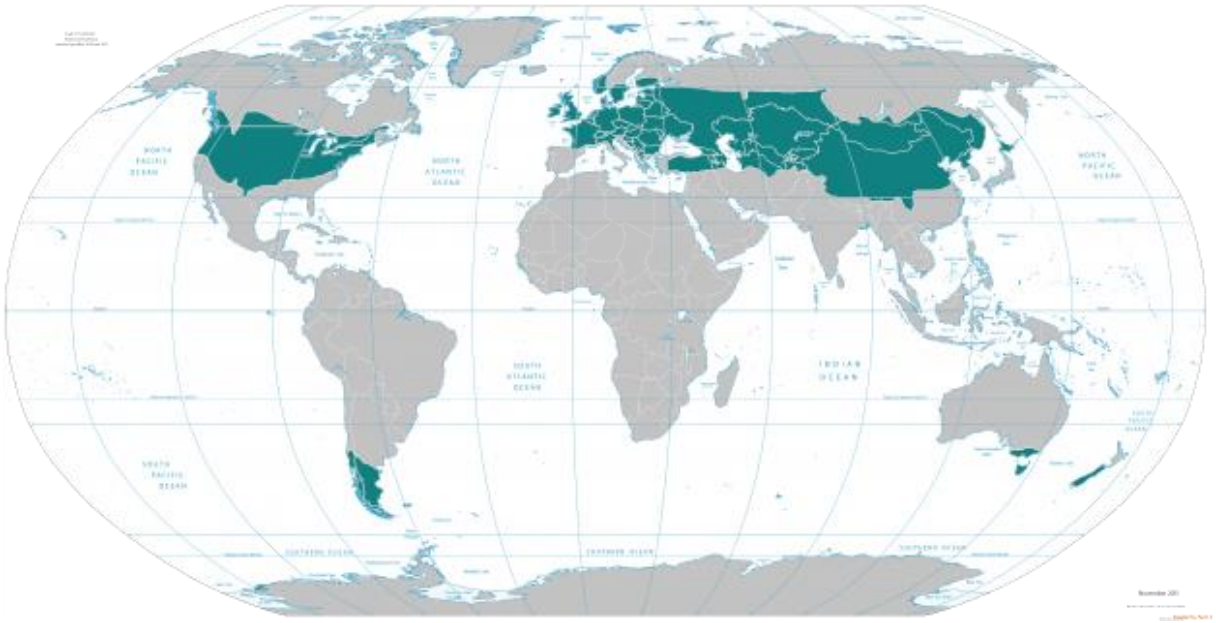

Figure 1 World map with temperate regions highlighted in green

In general, the main environmental signals that stimulate the establishment of dormancy are consecutive days with short photoperiod and/or low temperatures, although their degree of relevance depends on each species [2]. In *Betula* (birch), *Populus* (poplar) and *Salix* (willow tree) species, photoperiod is the main stimulus [6–8], while for *Malus* (apple), *Pyrus* (pear) and *Sorbus* (mountain-ash) species, low temperatures are more determinant [9,10]. Those differences in dormancy induction are related to the original localization of those trees when they developed this adaptation, mainly in the temperate regions of the world (Figure 1).

In trees growing in temperate conditions, temperatures decrease during autumn induces the physiological events leading to the entry into dormancy, while subsequent continuous exposure to cold during winter leads to its overcoming. Later, temperature increase in spring allows bud burst and flowering [9]. The perception of the first signs of cold induces biochemical and physiological modifications inside the buds that result in the establishment of the endodormant phase. In this phase, vegetative growth is inhibited until a sufficient amount of cold has been perceived, thus allowing the transition to the ecodormant phase. Upon reaching ecodormancy, the buds are able to burst after an adequate period of exposure to light and higher temperatures. Examples of buds displaying distinct phenological stages are presented in Figure 2.



Figure 2 Examples of apple tree bud phenology: variety of endodormant buds (up-left); silver-tip bud (down-left), green-tip buds (up-right), opened bud, pre-flowering (down-right).

To overcome the plant dormant cycle, *Rosaceae* temperate fruit trees require an appropriate chilling accumulation, which is measured as chilling units (CUs), defined as one hour at temperature ≤ 7 °C [11]. This amount of cold temperature is called chilling requirement (CR) and, once settled, allows the buds to transit to (eco) dormancy. This last stage requires a certain amount of warm temperatures (called heat requirement or HR) to reactivate growth and bud break. Therefore CR corresponds to the amount of chilling dose (i.e., integration of duration and amount of cold temperatures) required for endodormancy release [12], whereas HR represents the integration of time and amount of exposure to warm temperatures necessary to overcome ecodormancy. The whole process results in bud break, flowering, and later after bud break, pollination and fruit set to fructification. CR and HR are very variable among species and within a species among cultivars. It is thus assumed genetic factors are responsible for determining the timing of bud break [3,13,14]. The requirement for

CR and HR is a crucial choice for fruit tree farmers in temperate zones since they have to choose the right cultivar adapted to the right region based on climate constraints for local adaptation [15].

In the context of current global warming, the availability of chilling is predicted to become more and more irregular depending on the year. This can cause problems in the time of bud break, flowering synchronization, and consequently, the yield. In addition, higher temperatures registered in spring lead to early flowering and, therefore, increase the risk of flowers and fruit damage by spring freeze [16,17]. This threat concerns several countries worldwide where winter temperatures are mild and important fruit species are grown, for which temperature changes mainly trigger the dormancy cycle and flowering. Despite recent advances in winter bud dormancy understanding, some information is still lacking, in particular regarding the role of post-transcriptional and epigenetics regulation in the control of dormancy cycle (i.e., endodormancy, ecodormancy and bud break/flowering).

THE GENETICS OF BUD DORMANCY

The genetic studies of bud dormancy have started in two complementary directions, one deriving from the discovery of mutants with phenotypes linked to bud phenology, the second focusing on the analysis of large populations of hybrids or populations of diversity. In the first direction, the identification of the non-dormant phenotype of the evergreen mutant of peach (*evg*) has opened a new avenue for dormancy comprehension. This mutant does not present dormancy during winter. The *evg* mutant has a deletion in a genomic region that includes a group of genes encoding MADS transcription factors (TF) [18]. Those genes were then considered potential regulators of dormancy and then named *DORMANCY-ASSOCIATED MADS-box* (*DAM*) genes. DAM genes belong to the classical MIKC type of MADS TF and are similar to *SHORT VEGETATIVE PHASE* (*SVP*) from *A. thaliana* [19]. Characteristically, the expression of DAM genes from many fruit tree species, including apple (MdDAMs), displays distinct seasonal patterns [20]. A subgroup of DAM genes, including MdDAM1 from apple, is transcriptionally induced by low temperatures, and their maximum peak of expression occurs in the middle of the winter. This particular expression profile is believed to be linked to the physiological establishment and

maintenance of endodormancy [21]. The expression of these *DAM* genes gradually decreases during winter and reaches minimum values around the time when CR is satisfied and endodormancy is released (Figure 3) [20,22]. Besides *DAMs*, other genes encoding MADS TFs seem to have a role in the dormancy regulation of fruit trees. This is the case of homologs of the A. thaliana *SVP* and *FLOWERING LOCUS C* (*FLC*) genes (also named *SVP-like* and *FLC-like* genes, respectively). In apple, *MdFLC-like* mRNA peaks at the transition from endo- to ecodormancy and its function has been related to growth inhibition during ecodormancy (Figure 3) [23,24]. Two canonical *SVP-like* genes are present in the apple genome (*MdSVPa* and *MdSVPb*) [20,25]. The two apple *SVP-like* genes show constant expression levels during the dormancy cycle. Remarkably, the MdSVPa TF interacts with DAM and FLC-like TFs to form protein complexes in a sequential manner as a part of a gene regulatory network (GRN) that controls the dormancy cycle in the apple tree (Figure 3) [23].

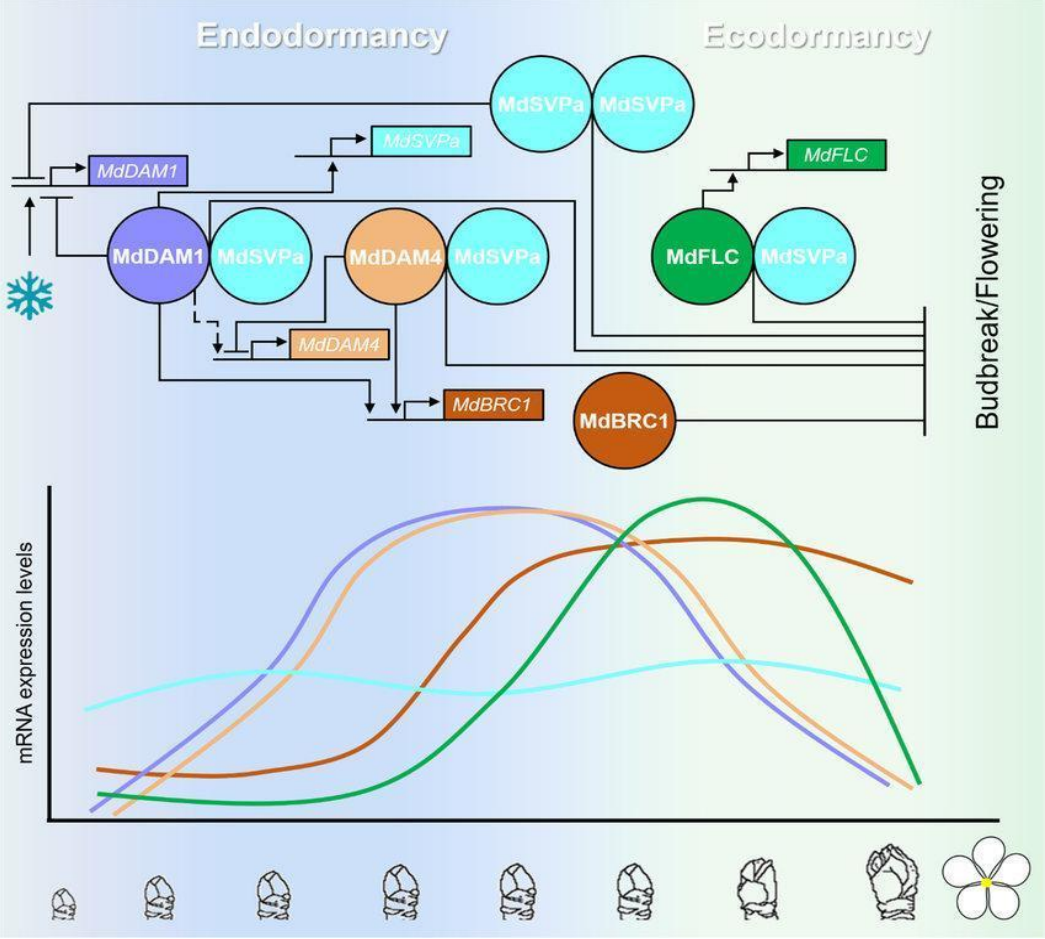

Figure 3 Proposed model from [23] summarizing the regulatory interactions between MADS transcription factors (TFs) during the apple tree dormancy cycle. Apple MADS TFs MdDAM1, MdDAM4, MdFLC and MdSVPa form complexes that regulate the expression of each other in a genetic regulatory network.

The study of plants misexpressing *DAM* and *SVP-like* genes evidenced their putative distinct function in growth cessation, bud set, bud endodormancy and bud break. For instance, the artificial silencing of *MdDAM1* alone or simultaneously together with *MdDAM4*, *MdDAMb*, *MdSVPa* and *MdSVPb* leads to a non-dormant phenotype in apple [26, 27], indicating their role in bud dormancy induction. Additionally, the overexpression of Japanese apricot (*Prunus mume*) *PmDAM6* gene in poplar induced growth cessation, bud set and bud endodormancy, while overexpression of *MdDAMb* and *MdSVPa* led to late bud break but no effect in the bud set [27, 28]. This indicates that *MdDAMb* and *MdSVPa* are likely more important in the late phase of endodormancy and/or ecodormancy. *SVP-like* genes have been studied not only in the cold-induced dormancy of *Rosaceae* species but as well in the light-induced dormancy in hybrid aspen, which presents three *SVP* instead of two like in apple, but the functions seem the same in the control of bud dormancy [29].

Other types of genetic studies have been devoted to quantitative genetic studies. Based on large populations, either segregating or with large diversity, the variations in the flowering time have been correlated to the variation of genetic markers located along with genetic maps. Such an approach has allowed the identification of quantitative trait loci (QTL) related to the dormancy cycle (flowering time) in many *Rosaceous* species: such as apple [14], pear (*Pyrus pyrifolia*) [30], peach (*Prunus persica*) [31], almond (*Prunus dulcis*) [32], apricot (*Prunus armeniaca*) [33] and sweet cherry (*Prunus avium*) [34]. Several of these studies have revealed *DAM* genes within the QTL intervals or close to, suggesting that those genes could also contribute to the within-species genetic variations of flowering time.

HORMONES and REDOX STATE in BUD DORMANCY

Hormones are major regulators of plant development and mediate signaling between organs or plant parts. The plant hormone gibberellin (GA), for example, is known to be involved in the control of flowering in *A. thaliana* [35]. More generally, GAs are known for their role in the promotion of growth and their signaling pathways are negatively regulated by DELLA proteins [36]. DELLA functions to block the transcriptional activity of TFs by physical interaction and as a transcriptional coactivator through interaction with another TF for DNA binding [37]. GA stimulates the degradation of DELLAs via the 26S proteasome by the formation of a complex with the

GA receptor GIBBERELLIN INSENSITIVE 1 (GID1). In the absence of GA, DELLAs accumulate and repress GA responses, for example, growth and floral induction [36,37]. A wide range of genes are GA-responsive and the control of GA activities at the organ level is done mostly by GA transport and specific organ accumulation [38–40]. Interestingly in fruit trees, GA has been associated with the promotion of dormancy release and induction of bud break. For example, the content of GA in pear buds is related to the timing of the endodormancy release and GA treatments can promote bud burst during endodormancy in Japanese apricot [41,42]. GA also regulates plant growth and development, including seed germination and bud dormancy [43,44]. It is believed that GA plays a key role in the release of dormancy in buds, but the molecular mechanism of gibberellin regulation is undefined.

Another interesting hormone is the abscisic acid (ABA), which is known as a major growth repressor and as well as stress-related signaling [45]. It was proposed the role of ABA-mediated growth cessation at the beginning of dormancy in response to photoperiod [46]. It is suggested that the accumulation of ABA in the buds triggers plasmodesmata closure, leading to the start of the endodormancy phase, while the GA signaling at the end of endodormancy would be responsible for reconnection of plasmodesmata communication and growth resumption. Endogenous levels of ABA increase at dormancy establishment and decline during the transition from endo- to eco-dormancy. This has been observed in many fruit trees and vines, such as grapevine (*Vitis vinifera*) [47], peach [46], pear [48,49], and sweet [50].

The opposite role of GA and ABA in activating and inhibiting growth during the dormancy cycle suggests the existence of a dual hormonal balance that governs dormancy-related phase switches (i.e., from endo- to eco-dormancy). Other hormones such as ethylene, auxin, cytokinin and jasmonates are also known to be involved in dormancy control of fruit trees (Reviewed by [51]) and are not included in this Introduction. It has been proposed that hormonal signaling (mainly ABA and GA) integrates into gene regulatory networks controlling the dormancy cycle in fruit species [45,52]. A summarized proposed model by [53] is presented in Figure 4.

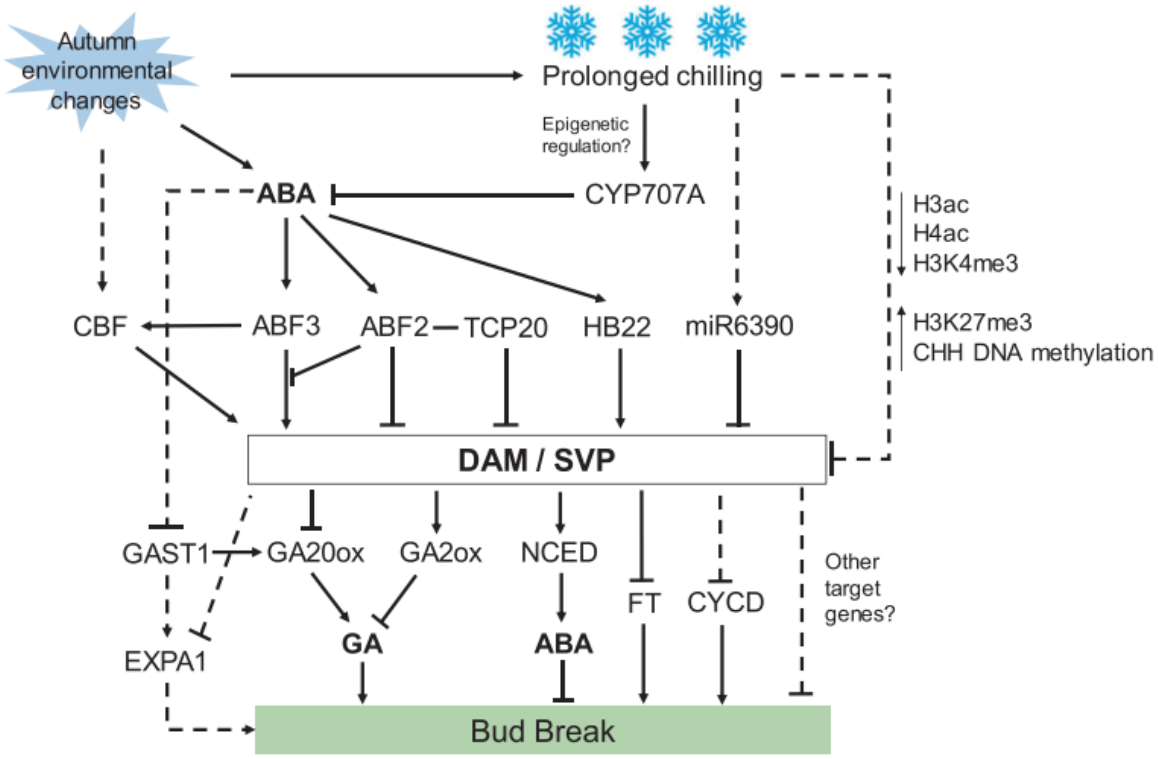

Figure 4 Proposed model from [53] integrating genetic regulation by environmental cues as temperature, hormonal influences and post-transcriptional regulation by miRNAs, as well epigenetics. This proposed model would be the base of the bud dormancy cycle.

In addition to hormones, other substances are being flagged as potential signaling molecules that participate in the control of bud dormancy in trees. This is the case of signaling molecules derived from the redox metabolism homeostasis seem to play a role in the endodormancy release, notably by acting in developmental switches modulated by the balance between GA and ABA [54]. Reactive Oxygen Species (ROS), in special hydrogen peroxide (H_2O_2), play a significant role in stress communication for adaptation to environmental changes [55,56]. This role has been extensively studied in the context of seed dormancy. For example, it was observed that the GA-ABA balance in apple dormancy seeds was potentially impacted by ROS signaling [54]. Furthermore, it was recently observed that the H_2O_2 accumulation patterns correlated with the dormancy stage and bloom time of two apple cultivars, with distinct bud break dates, peaking at the endodormancy release [57]. Further evidence of the role of ROS signaling in bud break comes from the study the more the classic dormancy breaker agent, hydrogen cyanamide (HC). Indeed, it has been suggested that HC causes bud burst even absence of a completed fulfilling of CR by activating

oxidative stress [58]. The precise mechanism of ROS action in bud dormancy of fruit trees, and in particular in the apple tree, remains unclear yet.

ARABIDOPSIS THALIANA as a MODEL for PLANT DEVELOPMENTAL STUDIES

A. thaliana is a largely used model species for dicotyledonous plants. Within the advantages of this plant model species, *A. thaliana* has a very small genome and short life cycle, as well as the availability of large collections of mutants, which have facilitated molecular investigations, especially regarding the control of flowering time. *A. thaliana* presents diverse pathways to achieve flowering in relation to distinct environmental and internal cues (e.g., photoperiod, temperature, gibberellin, sugars and age). Among them, the vernalization pathway is referenced as a model to study bud endodormancy in other species in which flowering is triggered by exposure to cold [59]. Vernalization (from Latin vernus, "of the spring") is the induction of flowering by exposure to the prolonged cold of winter [59,60]. In *A. thaliana*, the vernalization pathway controlling flowering time is regulated by a MADS-box transcription factor encoded by the *FLC* gene [61]. The floral repressor FLC inhibits the expression of *FLOWERING LOCUS T* (*FT*), which encodes a mobile signal that promotes flowering [60,62]. In plants exposed to cold conditions during winter, *FLC* mRNA levels are progressively reduced by an epigenetic mechanism that involves histone methylation of the *FLC* locus, releasing *FT* from its repression. In spring, *FT* mRNA is transcriptionally activated (by the long days and warm temperatures), allowing plants to flower [63]. It has been shown that FLC forms a protein complex with SVP to repress *FT* and the floral promoter gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), besides controlling the expression of several genes related to GA biosynthesis and signaling [62,64,65].

Many of the flowering pathways described in *A. thaliana* are regulated not only at the transcriptional levels but also post-transcriptionally. Specifically, flowering time is affected by mechanisms involving microRNAs (miRNAs) and Alternative Splicing (AS) regulation. MiRNAs are small RNAs varying from 20-24 nucleotides (nt) that are presented in a wide variety of ways to negatively regulate transcription [66,67]. The 20-22 nt miRNAs are mostly acting by blocking the translation of the messenger RNA (mRNA) or leading it to degradation, while 24 nt miRNAs are related to transcriptional

silencing of transposons and pericentromeric repeats via the RNA-directed DNA methylation (RdDM) pathway [66,68–71]. The main pathway that leads to the transition from juvenile phase to reproductive in *A. thaliana* is regulated by the miRNAs miR156 and miR172. MiR156 is highly expressed in young seedlings and its expression declines when the shoot develops. This allows the transcriptional activation of the *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) family, which are targets of the miR156 [70,72]. The miR156-*SPL3* module regulates *FT* expression to control *A. thaliana* flowering time in response to ambient temperature [70]. In contrast to miR156, the miR172 is a positive regulator in juvenile-to-adult developmental transition and has the opposite expression profile to that of the miR156 [73]. The targets of miR156, *SPL9*, and *SPL10*, directly promote the expression of miR172b, which indicates that miR172 acts downstream of miR156 to promote adult identity [73]. MiR156 and miR172 act in the thermosensory pathway to regulate ambient temperature-responsive flowering under non-stress temperature conditions. Indeed, the transgenic plants with overexpression of miR172 showed a temperature-insensitive early flowering [70,73].

Genes in high organisms have exons and introns, being the exons, the parts encoding the information to produce proteins. During the transcription, the introns are removed from the mRNA in a process called splicing. AS is a deviation from this, where different combinations of splice sites within a mRNA precursor (pre-mRNA) result in the production of a variably spliced mRNAs or isoforms [74,75]. AS can be caused by a skipped exon, exons reorganization, intron retention or even 3’ and 5’ splicing site variation, resulting in longer or shorter exons. The mechanism of AS has been shown to be involved in the thermosensory flowering pathway in A. thaliana. In *A. thaliana*, *FLOWERING LOCUS M* (*FLM*) mRNA undergoes AS depending on temperature and it can present two isoforms: *FLM-δ* and *FLM-β*. At low temperatures, *FLM-β* is more abundant and its protein product can form dimers with SVP to repress flowering. On the other hand, at warm temperatures, *FLM*-δ is more abundant and the dimer FLM-δ-SVP cannot bind to DNA to block flowering [76].

The similarity of the cold effect in the flowering control mediated by MADS-box genes between *A. thaliana* and fruit trees has been uncovered by hints of similarities in *SVP*, *FLC* and epigenetics functions [23,77–79]. Yet, the understanding of the genetic mechanisms regulating bud dormancy and their comprehension in other

species, especially in *Rosaceae* fruit trees, are in their infancy compared to *A. thaliana.* However, how much of the knowledge on flowering regulation of *A. thaliana* could be translated to other species in the context of bud dormancy understanding is an open question. In particular, studies on epigenetics, gene silencing by miRNAs, and AS, among other molecular mechanisms that regulate reproductive development, are not well described in perennial fruit trees yet. MiRNAs have been found in other tree fruits [71], which indicates a potential role also in apple bud dormancy. The understanding of gene regulatory networks controlling dormancy at the transcriptomic and post-transcriptomic level and in response to environmental inputs is essential for further agricultural development and adaptation of cultivars to a changing climatic world.

ROSACEAE

Taxonomy and origin

The family *Rosaceae* can be divided into three subfamilies [78]: *Spiraeoideae* (to which belong the apple, pear, cherry, plum species)*, Rosoideae* (with strawberry, rose flower, blackberries, etc.) and *Dryadoideae* (Dryas). The subfamily *Spiraoideae* includes the tribe *Pyreae* with Pyrus genus and *Malus* genera (among others). The *Malus* genus includes the common cultivated apple (*Malus x domestica* Borkh) (Figure 5) [80]. The apple tree is the fourth fruit tree in the world in productivity, according to the *Food and Agriculture Organization of the United Nations* (FAO, 2019). The modern apple tree is considered to originate from the Tian Shan Mountains, in central Asia, due to the genetic variability of apple cultivars there as well the presence of *Malus sieversii*, considered the wild progenitor (Figure 6) [81]. Apple is considered one of the oldest domesticated fruit crops in the world and the human migrations distributed the apple to Egypt, Greece and Italy (around 3000 BCE). In Europe, the apple developed as an important fruit and its cultivation has followed European colonialism around the world [82].

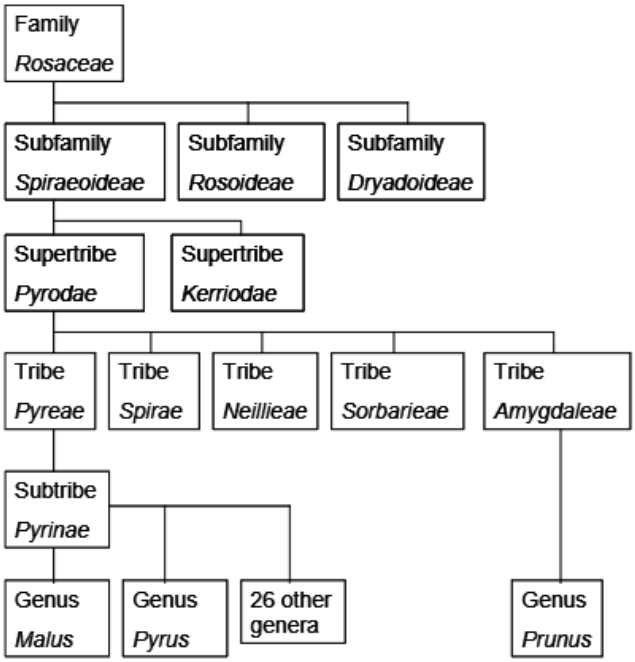

Figure 5 Taxonomy of fruit tree genera of Rosaceae family, with focus in Malus, Pyrus and Prunus, from [78]

The apple tree is a deciduous tree, which means that leaves are falling before winter. New leaves and flowering occurrence in spring are tightly connected to the dormancy cycle within the context of the annual cycle mediated by temperature. The size of a wild apple tree is almost 9 m, whereas in cultivated conditions, tree height is between 2 to 4 m. The limited tree growth is mostly due to dwarfing rootstock choices that facilitate tree training and management, associated with pruning practices and planting density [83,84].

The tree presents leaves in spiral phyllotaxy with serrate borders. The flowers are usually white or pink, with 3 to 4 cm in size, arranged in blooms of 4 to 6 flowers per inflorescence. The central flower of the inflorescence is called the "king bloom"; it opens first and can develop a larger fruit than lateral fruits within the cluster [85]. The apple fruit has an average size of 6 to 8 cm in diameter in commercialized cultivars. It presents a range of colors from green, yellow, pink to red and dark red. The fruit can be bi-color and its coloration can be smoothed with stripes of other colors. The fruits mature as soon as midsummer until late to mid-fall. The range of maturation time depends mostly on flowering bloom time, which in turn depends on dormancy release.

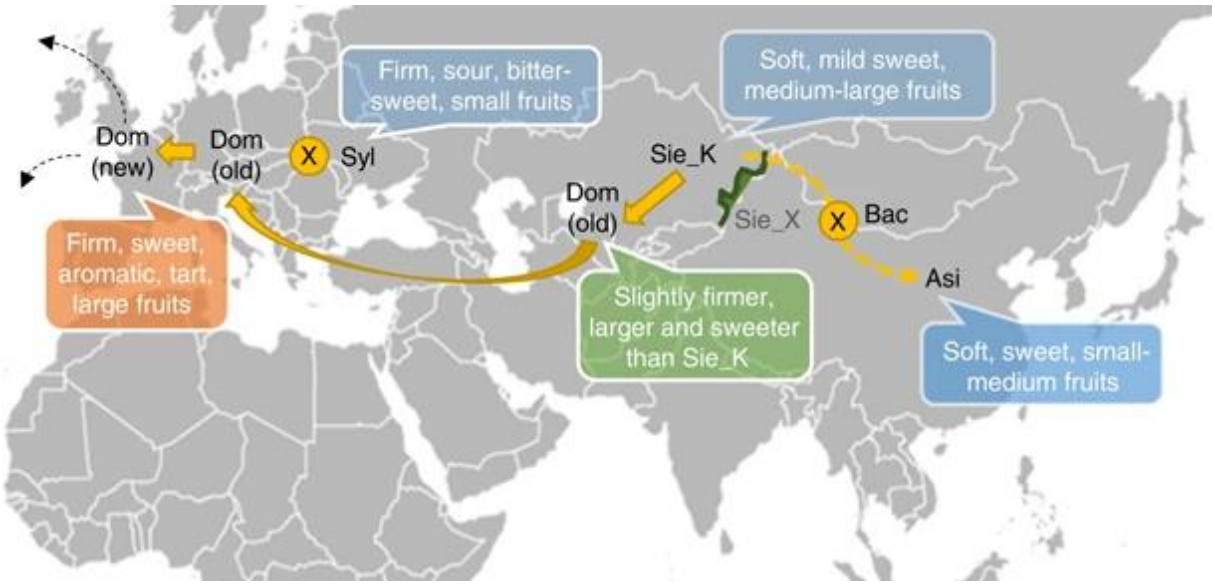

Figure 6 Origin of Malus species in central Asia and routes of dispersion during domestication until modern apple cultivars, from [81]

Genome and Genetics

The apple tree is a diploid (though triploid cultivars are not uncommon) plant. Its genome is composed of 17 chromosome pairs and the estimated genome size is approximately 651 Mb for the 'Golden double haploid' GDDH13 genome [86]. This genome was the most recently published when this Ph.D. research started and was used as a reference for this work. Previously, the first whole-genome sequence was obtained in 2010, based on the diploid cultivar 'Golden Delicious' [87]. The first whole-genome assembly was estimated to contain around 57,000 genes [87], though the most recent genome sequencing supports more moderate estimates of 45,116 protein-coding genes [87] or 44,677 [88]. Among other information, the availability of whole-genome sequences has provided evidence that the wild ancestor of the cultivated apple is most likely *Malus sieversii*. Re-sequencing of multiple accessions has supported this while also suggesting extensive introgression from *Malus sylvestris* following domestication [89].

The apple tree is an economically important tree [90] with defined growth and dormancy phases [91]. The apple tree is cultivated and consumed worldwide. The greatest productors countries are, in order: China, EUA and Turkey (FAO, 2019). Apple production also is present in countries with warmer winters, such as Brazil, South

Africa, and the ones in North Africa and the Middle East, where CR might not be fulfilled (Figure 7, from Our World in Data). This has generated the emergence of agronomic solutions such as the applications of chemicals (i.e., hydrogen cyanide that is not authorized in Europe). In the context of global warming, in which the chilling in winter is becoming less available and requires the use of chemicals and changes in management, this choice is becoming more decisive.

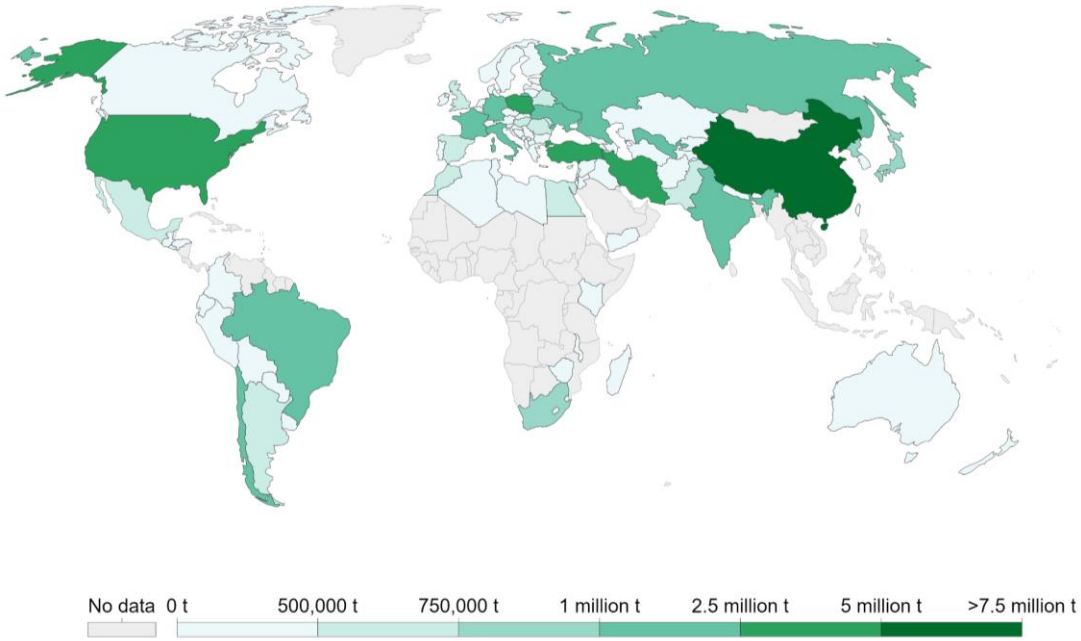

Figure 7 Apple production by country, measured in tons. Data FAO (2018) adapted from Our World in Data.

CLIMATIC CHANGES CONSEQUENCES

Nowadays, the understanding of dormancy has become more necessary than ever due to the perspective of global climate changes. Indeed, the adequacy of the plant's activity-dormancy cycle with local climatic conditions is essential for the survival of tree species in temperate and boreal conditions [92]. Negative consequences are predicted for agriculture in general, with unpredictable harvest or disrupted crop productions if new cultivars selection is not made for better adaptations to a warmer world [93]. The cycles of glaciation and heating are natural to our planet, but human activity has accelerated it at an unseen speed, and consequences are also expected to be unseen if nothing is done to adapt to a new world [94].

Actually, the increase in temperatures is expected to have antagonistic effects on bud break timing [16]. Indeed, less cold during winter will make it more difficult or

longer the time requested before endo-dormancy release. In contrast, warmer temperatures during spring will reduce the eco-dormant period and will accelerate bud break. These two effects do not generate similar risks and similar needs for new cultivars depending on the geographic regions. For instance, in the Mediterranean region, where the cold temperatures could become insufficient whereas eco-dormancy will be shortened, new cultivars are expected with low CR and still high HR. Another contrasting example would concern Eastern Europe, where the major risk is spring frost and the new cultivars would not necessarily request lower CR (as cold temperatures will certainly be high enough) but still higher HR to avoid spring frost. Therefore, the fine-tuning of either CR or HR or both will be necessary to adjust new cultivars to new climatic conditions.

The genetic control as well the role of hormonal signaling and epigenetics in the regulation of bud dormancy is starting to be elucidated. However, how the dormancy cycle is regulated at the post-transcriptional level by miRNAs and AS, and the conservation of this regulation in the model plant species *A. thaliana*, is completely unclear yet. Therefore, in this project, I intend to understand how genes involved in bud dormancy and bud break are transcriptionally and post-transcriptionally controlled by temperature. For this purpose, I used a transcriptomic approach to study the genes, AS isoforms and miRNAs potentially associated with bud dormancy control, especially at endodormancy release, considered as the critical point of dormancy. In the next chapters, I will discuss the genes and AS isoforms modulating expression during bud dormancy in contrasting cultivars (Chapter I). In Chapter II, I will characterize the transcriptional response of a set of candidate genes to changes in temperature and/or ROS and their potential association with endodormancy release. Chapter III corresponds to a published article in which I propose the role of miR159 in the endodormancy release in apple trees in relation to the sensitivity to ABA. Finally, in the General Discussion, I will explore the general advances that my thesis brings to the knowledge so far and perspectives of research in bud dormancy, as the gradual release of endodormancy, the potential role of *SVPa* isoforms and miRNAs in endodormancy release under stressful condition.

OBJECTIVES

The general objective of this Ph.D. project was to understand the mechanism used by fruit trees to sense and respond to changes in temperature to overcome endodormancy and initiate bud break. The two corresponding stages, i.e., endo- and eco-dormancy, were considered as the two most critical limiting steps. To achieve this purpose, I used the apple tree as a model plant and pursuit these three sub-objectives:

(1) Identification and characterization of genes, alternatively spliced isoforms, and miRNAs potentially involved in bud dormancy and bud break of apple trees by transcriptomics approach.

(2) Analysis of the effects of different temperatures and oxidative stress treatments on the expression levels of candidate genes and alternatively spliced isoforms in the cultivar 'Golden'.

(3) Characterization and comparison of the expression levels of candidate genes between two apple cultivars showing contrasted chilling requirements for endodormancy release.

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CHAPTER 1

Transcriptomic and post transcriptomic changes associated with bud dormancy in the apple tree

ABSTRACT

Dormancy is an adaptive mechanism that enables plants to survive unfavorable climatic conditions and allows bud break and flowering to occur only when the conditions are permissive. This resting stage is defined by the absence of visible growth. In apple (*Malus domestica* Borkh.) and many other Rosaceae species, winter bud dormancy can be divided into at least two phases: endo- and eco-dormancy. During endo-dormancy, growth repression is controlled by internal cues and it is released by long periods of cold (chilling requirement = CR). Then, buds undergo an ecodormancy phase and become competent to bud break in response to warm temperatures. In the model plant species *Arabidopsis thaliana*, changes in ambient temperature regulate flowering time through mechanisms that involve the alternative splicing (AS) of MADS-box genes. I have hypothesized that a similar mechanism could operate to control the temperature-dependent transition between endo- and eco-dormancy phases in the apple tree. In order to address this question, we have performed a RNA-seq time-course experiment on buds of the apple tree (cv. *Golden delicious*) during the dormancy cycle. RNA from time-points covering the endo- to eco-dormancy transition were sequenced and changes in gene expression and AS were quantified. My bioinformatics analyses showed that pathways related to chromatin silencing, temperature sensing, flowering, ROS signaling and hormones, among others, were affected during the transition between endo- to eco-dormancy. I have observed an important shift in gene expression at the endodormancy release. Indeed, more than 10000 genes were differentially expressed (log2FC ≥ 1, FDR ≤ 0.05) between the first and last time point (endodormancy and ecodormancy) and characteristically, most of them were upregulated. I have also found that cultivars with distinct CR presented the same pattern of expression of the *DORMANCY ASSOCIATED MADS-BOX* (*DAM*) genes but a differential expression for the apple homologs of the *A. thaliana* flowering-related *HOMEOBOX PROTEIN 22* (*MdHB22*) and *AINTEGUMENTA* (*MdANT*) genes.

Moreover, I have implemented a bioinformatics pipeline to identify differential alternatively spliced genes associated with the endo- to eco-dormancy transition. My preliminary analysis identified a potential AS of the dormancy-related *Malus domestica* (*Md*) gene *SHORT VEGETATIVE PHASE a* (*MdSVPa*), a gene that

operates together with the *DAM* genes in the dormancy regulation. This isoform presents a shorter C-terminal motif that could interfere with its ability to form dimer/tetramer and, thus, with its role in dormancy and bud break control.

In this Chapter, I show the transcriptomics analyses used to isolate candidate genes potentially involved in dormancy-cycle control and to describe mechanisms of post-transcriptional regulation involved in fruit tree development.

RÉSUMÉ

La dormance est un mécanisme adaptatif qui permet aux plantes de survivre à des conditions climatiques défavorables et permet le débourrement et la floraison de se produire uniquement lorsque les conditions sont permissives. Cette étape de repos est définie par l'absence de croissance visible. Chez le pommier (*Malus domestica Borkh.*) et de nombreuses autres espèces de Rosacées, la dormance hivernale des bourgeons peut être divisée en au moins deux phases : endo- et éco-dormance. Pendant l'endo-dormance, la répression de la croissance est contrôlée par des signaux internes et elle est libérée par de longues périodes de froid (besoin de refroidissement = CR, ou en Anglais Chilling Requirement). Ensuite, les bourgeons subissent une phase d'écodormance et deviennent compétents pour le débourrement en réponse aux températures chaudes. Chez l'espèce végétale modèle *Arabidopsis thaliana*, les changements de température ambiante régulent le temps de floraison par le biais de mécanismes impliquant l'épissage alternatif (AS, Alternative Splicing) des gènes MADS-box. J'ai émis l'hypothèse qu'un mécanisme similaire pourrait fonctionner pour contrôler la transition dépendante de la température entre les phases d'endo- et d'éco-dormance chez le pommier. Afin de répondre à cette question, nous avons réalisé une expérience de séquençage d'ARN pendant un court laps de temps sur des bourgeons de pommier (cv. Golden delicious) pendant le cycle de dormance. Les ARN des bourgeons échantillonnés aux dates couvrant la transition endo- à éco-dormance ont été séquencés et les changements dans l'expression des gènes et l'AS quantifiés. Mes analyses bioinformatiques ont montré que les voies liées à l'extinction de la chromatine, à la détection de la température, à la floraison, à la signalisation ROS et aux hormones, entre autres, étaient affectées lors de la transition entre l'endo-endormance et l'éco-dormance. J'ai observé un décalage important de l'expression des gènes lors de la libération de l'endodormancie. En effet, plus de 10 000 gènes ont été exprimés de manière différentielle (log2FC ≥ 1, FDR ≤ 0,05) entre le premier et le dernier point dans le temps (endodormance et écodormance) et, de manière caractéristique, la plupart d'entre eux ont été régulés à la hausse. J'ai également découvert que les cultivars avec une CR distincte présentaient le même schéma d'expression des gènes *DORMANCY ASSOCIATED MADS-BOX GENES* (*DAM*),

mais une expression différentielle pour les homologues de pomme de la *HOMEOBOX PROTEIN 22* (*MdHB22*) liée à la floraison d'*A. thaliana* et *AINTEGUMENTA* (*MdANT*).

INTRODUCTION

Temperate fruit trees face significant seasonal variations, especially in environmental factors such as temperature, day length, and water availability that have a tremendous effect on yield production. In response to this climatic context, trees have developed several adaptive strategies. One of those strategies is the bud dormancy, a mechanism designed by perennial plants growing in temperate climates to protect their meristems from the low temperatures (e.g., frost) during winter, and it is associated with a temporary suspension of growth [1,2]. In autumn in temperate climates, low temperatures and short photoperiods induce growth cessation in all shoot apical meristems (SAM) and the formation of winter buds that protect the meristematic tissues [3]. Then SAM transits through distinct phases of the dormancy cycle that ends in bud break. The dormancy cycle can be divided into three stages according to the growth inhibition mechanism: paradormancy, endodormancy, and ecodormancy. Paradormancy (or correlative inhibition) consists of the growth inhibition driven by another plant organ, such as apical dominance. Endodormancy is the inability to grow in favorable conditions (or 'true dormancy'), whereas ecodormancy is the growth inhibition by unfavorable conditions [1,2]. The winter bud dormancy cycle often refers to the endodormancy phase and, sometimes, it also comprises ecodormancy if the weather is still unfavorable after endodormancy finishes [4]. For the *Rosaceae* family, which includes the most economically crucial temperate fruit crops, chilling is the primary environmental factor, followed by light changes, promoting endodormancy set and release [5,6]. The most prominent *Rosaceae* cultivated species is the apple tree (*Malus domestica* Borkh.), being the fourth fruit crop in the world in productivity, according to the *Food and Agriculture Organization of the United Nations* (FAO, 2019). In the apple tree, the drop in daily mean temperatures induces dormancy, with the encapsulation of the apical meristem by scales. Once settled, the (endo) dormancy requires a period of chilling (chilling requirement or CR) to transit to (eco) dormancy, and this last stage requires warm temperatures (heat requirement or HR) to reactivate growth and to bud break. Therefore, CR corresponds to the amount of chilling dose (i.e., integration of duration and cold temperatures) required for endodormancy release [7]. In contrast, HR represents the amount of exposure to warm temperatures necessary to overcome ecodormancy (after endodormancy),

resulting in bud break, flowering, and fructification. Thus, the variation of quantity and intensity of the cold and heat are the primary triggers of dormancy transitions and release. CR and HR are very variable among species and cultivars, being genetic factors the most responsible for determining this characteristic [1,8,9]. Commonly used cultivars such as Golden delicious and Pink Lady, as well as their close relatives, display moderate (~1000 Chilling Hours [CH]) and low (~400 CH) chilling requirements, respectively. Therefore, these cultivars can be used in breeding programs to adapt apple cultivars to local climatic conditions.

In Rosaceae species, it is believed that a group of genes encoding MADS transcription factors (named for original members MCM1 (*Saccharomyces cerevisiae*), AGAMOUS (*A. thaliana*), DEFICIENS (*Antirrhinum majus*), and Serum response factor (*Homo sapiens*) are central regulators of the dormancy cycle. These genes are referred to as *DORMANCY-ASSOCIATED MADS-BOX* (DAM) genes. They were first identified because of their genetic association with the non-dormant phenotype of the *evg* mutant of peach [10]. In apple, phylogenetic analyses showed that *DAM* genes are similar to *SHORT VEGETATIVE PHASE* (*SVP*), which encodes a MADS transcription factor that acts as a floral repressor in *A. thaliana* [11,12]. Further genetic studies have identified *DAM* genes within QTLs related to the dormancy cycle in diverse fruit tree species, such as apple [8], pear (*Pyrus communis L*.) [13], peach (*Prunus persica*) [14,15], almond [Prunus dulcis (Miller) D. A. Webb] [16], apricot (*Prunus armeniaca L*.) [17] and sweet cherry (*Prunus avium*) [18]. *DAM* genes from several fruit species such as apple, pear, peach, sweet cherry, and kiwi (*Actinidia deliciosa*) fruit display distinct seasonal patterns with maximum peaks of expression at different times during the dormancy cycle, suggesting a role in regulating specific dormancy phases (e.g., endodormancy and ecodormancy) [3]. In apple, the expression of particular *DAM* genes (*DAM1* and *DAM4*) is up-regulated by low temperature and shows a maximum peak of expression in the middle of the winter. This peak of expression has been associated with the physiological establishment and maintenance of endodormancy [19]. Characteristically, the expression of these *DAM* genes progressively decreases after long exposure to cold and reaches its minimum around the time when CR is satisfied and the breakage of endodormancy occurs [20,21]. It has been proposed that two mechanisms of cold sensing and signaling

operate during the temperate tree dormancy cycle to control *DAM* expression [22]. A group of *DEHYDRATION-RESPONSIVE ELEMENT BINDING* (*DREB*) protein/C-repeat binding factors (CBFs) seems to play a role in direct response to cold by activating the transcription of some *DAM* genes, whereas the gradual silencing of *DAM* genes upon exposure to low temperature might be epigenetically regulated by an unknown mechanism [3,22].

Furthermore, several transcriptomic studies performed on fruit tree species identified other dormancy-related genes based on their expression profiles during dormancy and bud break that are similar to those of the genes regulating the vernalization pathway in *A. thaliana* [14,23]. A gene similar to the *A. thaliana FLC* (called *MdFLC-like*) has been identified in two transcriptomic studies as a potential apple bud dormancy regulator [23,24]. Remarkably, *MdFLC* mRNA is induced by cold during the endodormancy phase and its expression decays during ecodormancy in response to warm temperature [25].

Several genetic and functional studies performed in the model plant *A. thaliana* have elucidated the genetic control of flowering time by environmental cues [26]. In this plant model, there have been defined several pathways that control flowering time: photoperiodic, autonomous, gibberellin, vernalization, age, sugar, and thermosensory [27–30]. In terms of the thermosensory pathway, it has been shown that in *A. thaliana*, cold temperatures delay flowering. In contrast, warm temperatures accelerate it by mechanisms that involve post-transcriptional regulation, and in particular, the alternative splicing (AS) of genes encoding MADS transcription factors [27,31–33]. *SVP* plays a central role in thermo-responsive flowering, as well as in vernalization, by repressing the mRNA abundance of the floral integrator genes *FLOWERING LOCUS T (FT*) and *SUPPRESSOR OF CONSTANS 1 (SOC1)* [34–36]. In *A. thaliana*, it has been proposed that SVP forms floral repressor complexes with the MADS-box transcription factors *FLOWERING LOCUS M* (*FLM*) and *MADS AFFECTING FLOWERING* 2, 3, and 4 (*MAF2-MAF4*) at low temperatures [32,37]. Notably, *FLM* is alternatively spliced, and distinct *FLM* isoforms are preferentially accumulated at contrasting temperatures. The isoform *FLM-β* is predominantly expressed at low temperatures, and its protein product forms a complex with SVP that represses floral induction [32]. However, at warm temperatures, the *FLM-β* isoform is low expressed, and other isoforms, including

FLM-δ, are predominantly transcribed. Conversely, warm-transcribed *FLM* isoforms contain a premature stop-codons that promotes their post-transcriptional downregulation by a mechanism of alternative splicing coupled with nonsense-mediated mRNA [27,38]. In the absence of FLM, SVP cannot repress *FT* and *SOC1*, allowing the acceleration of the floral transition at warm temperatures [27].

AS is a conserved post-transcriptional mechanism across eukaryotic species, including perennial plants, which regulates – among other biological processes – plant development in response to environmental factors [39,40]. Furthermore, AS also modulates temperature responses in woody plant species that experience winter bud dormancy, such as grapevine [41] and pear [42]. Thus, changes in ambient temperature can be expected to regulate bud dormancy and flowering phenology in perennial fruit trees through molecular mechanisms mediated by AS. However, the available information regarding the identity and the mode of regulation of the genes acting in the temperature-mediated control of bud dormancy and bud break processes is still limited.

In the context of global warming, the predicted irregular chilling availability can cause problems in the time of bud break, flowering synchronization, and consequently, the yield, whereas higher temperatures registered in spring lead to early flowering and, therefore, fruit damage by spring freeze [43,44]. This threat concerns several countries worldwide where winter temperatures are mild and some other essential fruit species, where temperature changes mainly trigger the dormancy cycle and flowering disruption. Despite the recent progress, our understanding of how winter bud dormancy is controlled in fruit trees remains fragmentary [3]. Notably, there is not a clear picture of the integration of genetic circuits and hormonal signaling in the environmental control of endodormancy, which has a huge impact on the timing of bud break.

In this Chapter, I aim to identify genes potentially involved in the control of dormancy release of the apple tree by using RNAseq. These transcriptomic studies will be used to select genes differentially expressed during dormancy that could play a role in the control of endo- and eco-dormancy phase transition. A subset of these genes might explain the distinct dormancy and flowering behaviors of contrasted apple cultivars. Finally, I will perform a preliminary analysis to detect AS

events that will pave the way to a better understanding of the post-transcriptionally regulation of dormancy in the apple tree.

2 MATERIAL and METHODS

2.1 Plant Material

Shoots and flower buds were collected every two weeks from October until March in the 2018/2019 and 2019/2020 cycles. Two cultivars with contrasting flowering time and CR were harvested: Pink Lady (hereafter PL) and Golden delicious (hereafter GD), being an early flowering and a midi-late flowering cultivar, respectively. The trees were grown under the same conditions in an orchard based in the SudExpe field (Marsillargues – France, 43°63'26'' N, 4°16'39'' E).

For the forcing tests, several branches of 30 to 40 cm of at least two years and containing approximately 10 terminal floral buds were collected at each date. The branches were transported to the laboratory directly from the sampling for phenological determination. For gene expression studies (RNA-seq and RT-qPCR), three buds from short shoots (spurs of more than two years) per tree and date were collected. A total of nine buds from three trees were pooled together to compose an independent biological sample. In this way, three biological replicates from the same nine trees were collected at each date. Buds were frozen in liquid nitrogen right after the sampling and stored at -80°C in the laboratory. Buds from the apple cultivar Golden delicious collected between 2018 and 2019 were used for the RNA-seq, while buds from Golden delicious and Pink Lady cultivars from two consecutive winter seasons (2018/19 and 2019/20) were collected for RT-qPCR analyses.

2.2 Determination of the phenological stages

For the Tabuenca test [45], five flowering buds from the branches and three replicates per cultivar were tested (Figure 1a). This test is based on the capacity of a floral bud to grow and increase its weight (first indication for meristem reactivation) upon exposure to "forcing" conditions (i.e., 25 °C and photoperiod of 16h/8h light/dark) if the endodormancy is already released. Thus, it is possible to determine when the endodormancy is released by comparing the weight of buds collected on a given day to the bud weight of shoots harvested the same day and exposure to warm temperature for an additional week. The bud break test used four replicates of 10 single-bud cuttings

per cultivar that were put in water pots in forcing conditions for 30 days (Figure 1b). The percentage of buds that breaks during this period is used to calculate their dormancy status (i.e., 0% = completely dormant; 100% = fully active). The accumulation of chilling hours was calculated as the integral of hours above 7.2 °C.

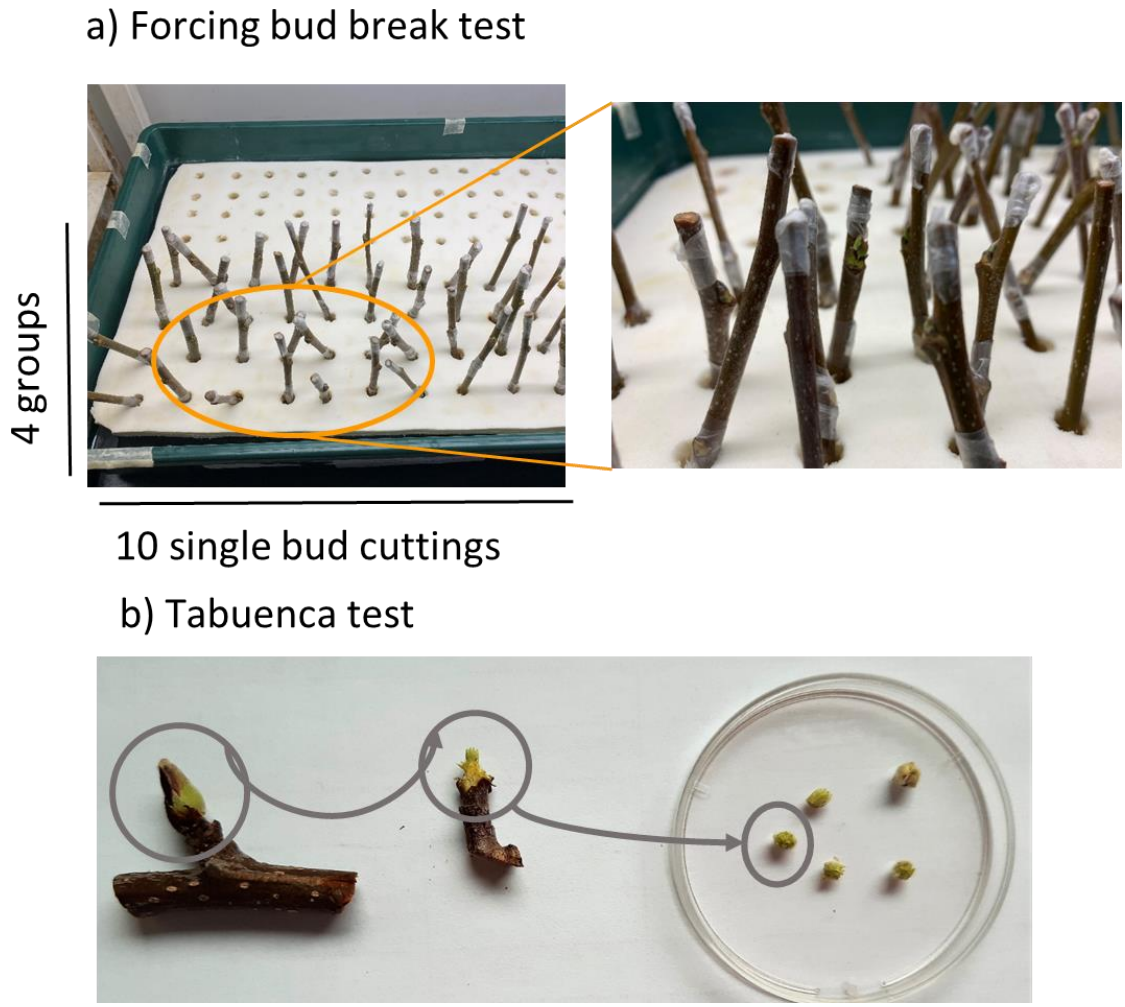


Figure 1-1 a) Forcing bud break test used to measure the time to bud bread (in days) and the percentage of bud break after 30 days of exposure to forcing conditions (25 °C and photoperiod of 16h/8h light/dark) This test was applied to 40 single bud cuttings. b) The Tabuenca test uses 3 of 5 apical floral meristems. Their fresh weight (without scales) is measured. Another set of buds are exposed to the same forcing conditions and measured after one week, as decribed in a).

2.3 RNA extraction

The samples previously selected based on their phenological state had their total RNA extracted from ~100 mg bud powder with 'Spectrum Plant Total RNA Kit' from Sigma for total RNA sequencing. The quality of the RNA was verified in a TapeStation 4200 (Agilent Genomics). Only RNA with RIN (RNA Integrity Number) over 8.5 (annex A) was used in the subsequent procedures.

2.4. RNA Sequencing and bioinformatics.

2.4.1. RNA sequencing

At least 40 µl of RNA at around 200 ng/µl (~ 8 µg) per sample were shipped at -20 °C on carbonic ice for RNA sequencing at the Sequencing Core Facility at Max Planck-Genome-Centre (Cologne, Germany). The RNA was treated for rRNA depletion before sequencing to obtain a high number of relevant reads. The sequencing was done on an Illumina HiSeq3000 instrument until a minimum of 15 million reads per sample using a 150 bp paired-end configuration.

2.4.2. Bioinformatic analysis of RNAseq data

The RNA-Seq data were analyzed in a partnership with the EMBRAPA's bioinformatic group, inside a *Laboratoire International Associé* (International associated laboratory - LIA) between INRAe (France) and EMBRAPA (Brazil).

2.4.2.1 Alignment to genome

The quality of the reads was assessed using FastQC [46], and possible adaptor contaminations were identified and removed using cutadapt software [47]. The data was also cleaned from rRNA,tRNA, ncRNA and sRNA based on references from the Rfam database, a multi-specie library covering non-messenger RNAs [48]. This was done to enrich expressed genes. Reads were then aligned on the last version of the reference apple genome, available in 2019 (GDDH13 v1.1 [49]) using the Kallisto software (https://www.kallistobus.tools/) [50].

2.4.2.2 Differential expression analysis

Differential gene expression quantification was done with three commonly used software: edgeR likelihood, edgeR quasi-likelihood, and NOISeqBIO. EdgeR implements a range of statistical methodology based on the negative binomial distributions, to model the read counts for each gene in each sample [51]. The quasi-likelihood functions provide more robust and reliable error rate control when the number of replicates is small [52]. The Likelihood was a novel empirical Bayes method that allowed each gene to have its own specific variability, even when there were relatively few biological replicates [53]. NOISeqBIO combines the non-parametric framework of NOISeq with an empirical Bayes approach. This method assumes that genes can be classified into two different populations; genes with an invariant expression between two experimental conditions and genes whose expression

changes between conditions [54]. The result obtained with each of the tested software was compared to select the one showing the highest number of differentially expressed genes (DEG) in common with the other two ones.

EdgeR was used for identification of DEGs (FDR < 0.05, Log2FC > 2) while DESeq2 [55] was used for clustering and gene expression visualization (p-value adjusted or FDR < 0.05). Both present similar results for DEGs identification by using the same negative binomial test, but EdgeR was used for the first part of the bioinformatic analysis when I was under the supervision of the EMBRAPA team, and I used DESeq2 when the work was done by my own as it is more user friendly.

2.4.2.3 Identification of Differentially Alternatively Spliced Isoforms (DASIs) during bud dormancy

The raw data from the RNA-seq was re-analyzed using the Tuxedo pipeline in order to identify and quantify AS events associated with the dormancy cycle [56]. The RNA-seq data was processed according to the default protocol (shown in Figure 1 of annex 5). TopHat2 was used to perform the read alignments, CuffLinks for the assembling and CuffDiff to calculate the differential expression between isoforms. Tophat is a splice-aware aligner, making this the main feature of this method for the analysis of isoforms. It aligns the reads (via bowtie) and also attempts to split alignments around splice sites. A regular aligner (as Kallisto or Hisat2) would classify these reads that do not align as ‘unfit’ rather than reads that are split across a splice junction. The identification of DASIs was made by comparing the second, third, and fourth data points against the first one (ENDO1/ENDO2, ENDO1/ECO1, ENDO1/ECO2).

2.4.2.4 Gene expression profiles clustering

Using DESeq2, genes differentially expressed between at least two time-points were selected. The threshold for this selection was a false discovery rate (FDR) < 0.05. The Benjamini–Hochberg method that controls the False Discovery Rate (FDR) using sequential modified Bonferroni correction for multiple hypothesis testing was used [54]. The gene expression profiles were clustered with the 'hcluster' R package using the 'complete' method for ranking, an adaptation of Ward’s Minimum Variance method that resulted in sequential clustering until a final cluster englobing all the genes. Then the groups formed were plotted using 'ggplot'.

2.4.2.5 Gene ontology enrichment analysis.

The Bingo Java-based tool [57] implemented into the Cytoscape program (v3.8.2, [58]) was used for Gene Ontology enrichment analyses on DEG (FDR < 0.05% and logFC< 2.5) between the comparisons among all the time-points and for the genes included in the clusters (FDR < 0.05%). Genes that presented enrichment in specific pathways were selected for further procedures.

2.4.3 Expression analysis by RT-qPCR

2.4.3.1 cDNA production

A total of 500 ng of RNA of samples diluted in 8 µL water and treated with 'RQ1 RNase-Free DNase' (Promega) was used to prepare cDNA. The reverse transcription was done using 'SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase' (ThermoFisher). Both procedures were done according to the manufacturer's instructions.

2.4.3.2 RT-qPCR

Quantitative PCRs were performed using the kit' LightCycler® 480 SYBR Green I Master' in the 'LightCycler 480 - Multiwell Plate 384' (Roche Diagnostics GmbH) according to the manufacturer's instructions. Primers were designed with the program Primer3 [59] (the table of primers used is in Annex A). The genes *MdMDH* and *MdWD40* were used as a reference for genetic expression calculus.

3 RESULTS

3.1 Physiological characterization of the phenological stage of two apple cultivars

The main objective of this Chapter is the identification of genes differentially expressed during the transition from endo- to eco-dormancy. This study was performed using GD, which is one of the most economically important apple cultivars and its genome is fully available [49]. We have also included the cultivar PL for comparative purposes, as it is known to satisfy endodormancy and undergo ecodormancy earlier than GD. In order to determine the phenological state of apple buds during the dormancy cycle, two forcing tests were done: Tabuenta and bud break tests (see Figure 1). The bud break test done on GD (Figure 2a) shows a decrease in bud burst percentage at the end of November, indicating the entrance in dormancy at this time of the year. GD buds recovered their growth capacity in the middle of February, the

moment at which an increase in the bud burst percentage was observed. A punctual and transitory bud growth reactivation was observed in January 2019. This phenomenon was probably caused by an unexpected pruning done a week before the measurement, and therefore, it was not considered for further analyses. PL buds only showed a slight decrease in bud burst rate from the middle of December until the middle of February, indicating that this cultivar is not experiencing a complete entrance into dormancy as GD does (Figure 2a,b). An estimation of the timing of the transition from endodormancy to ecodormancy phases is measurable by the Tabuenca test (Figure 3a,b). In the 2018/19 winter, PL transitioned from endodormancy to ecodormancy between the 15 and 21 of January 2019, corresponding to an accumulation of 450 CH (Figure 3c), while GD did it between 4 and 12 of February 2019 (Figure 3a), corresponding to an accumulation of 960 CH (Figure 3c). For 2019/20 winter, the transition happened between 5 and 12 of February 2020 for GD (700 CH) and between 10 and 17 January 2020 for PL (Figure 3b), with 400 CH for PL and 700 CH for GD (Figure 3d). The recorded flowering dates were 25 March 2019 and 24 March 2020 for PL, and 06 April 2019 and 11 April 2020 for GD.

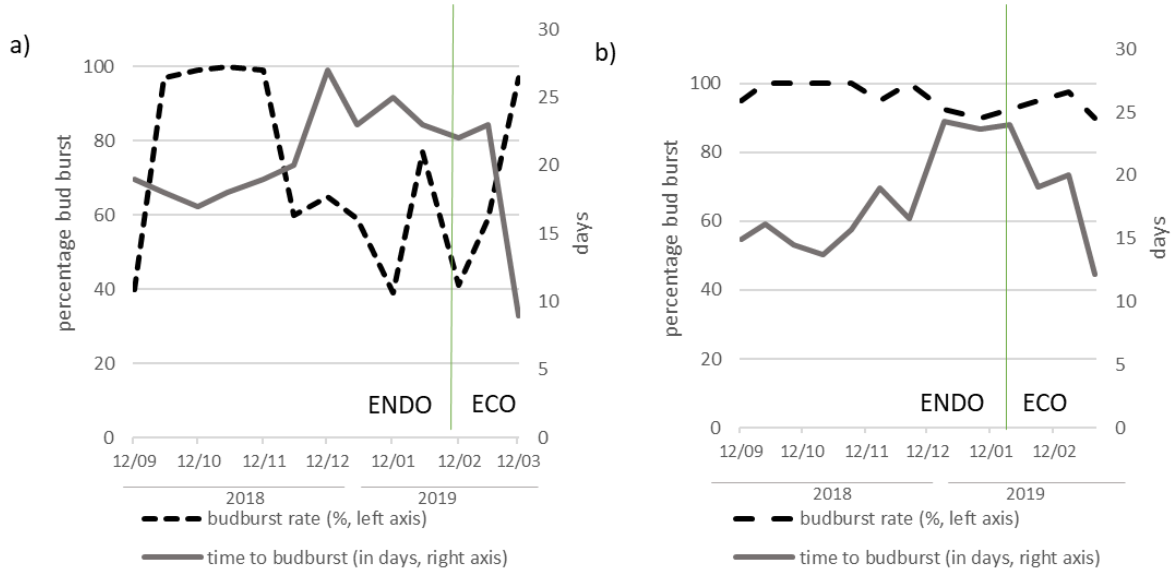


Figure 1-2 Bud burst test applied to Golden delicious (a) and Pink lady (b) cuttings during 2018/2019 winter dormancy cycle. Bud burst rate are represented by dashed lines and time to bud burst are represented by gray lines. Variables are expressed in percentage on the left y-axis and in days on the right y-axis.

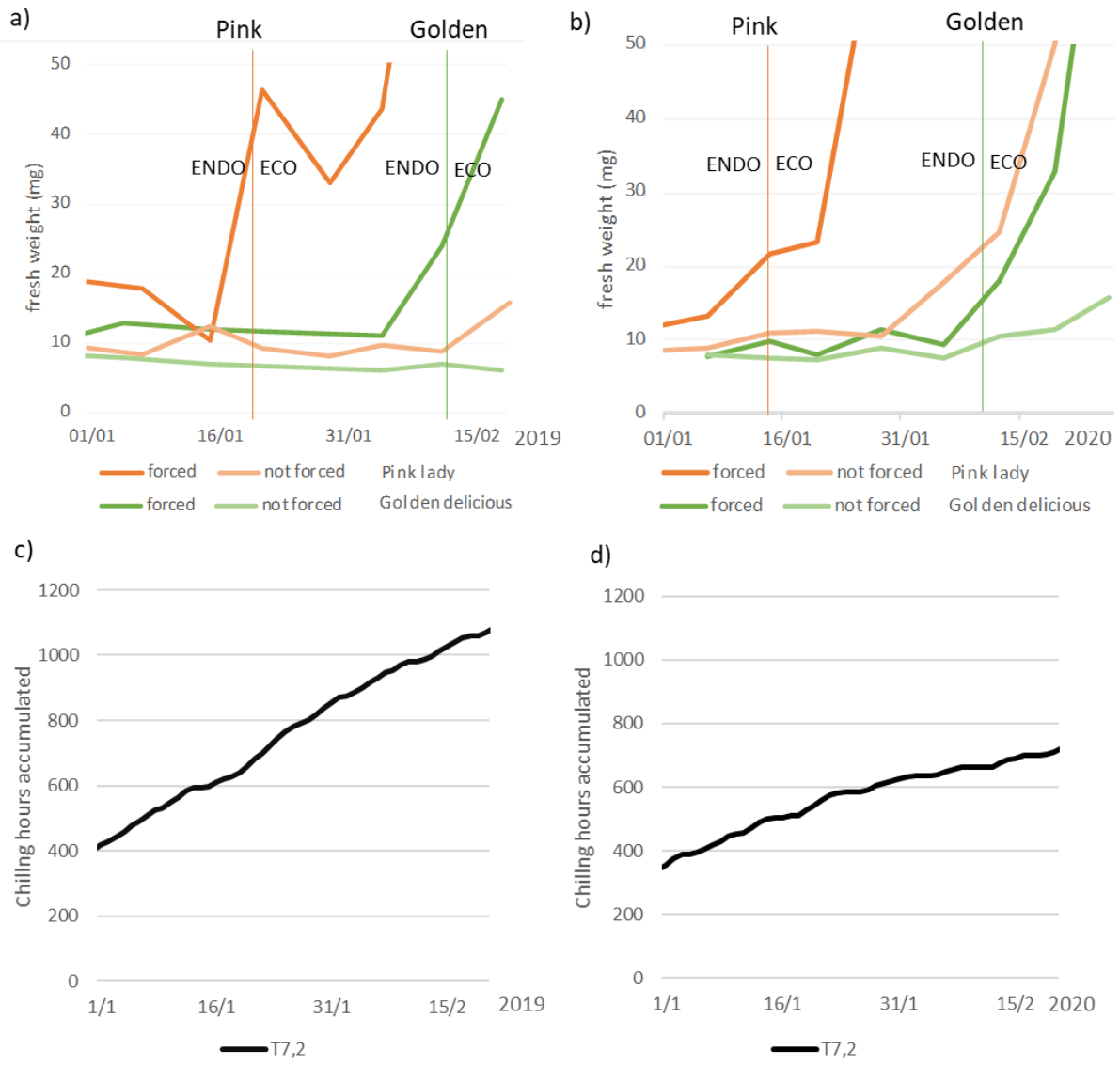


Figure 1-3 Fresh weight of Golden delicious (green lines) and Pink lady (orange lines) buds during Tabuenca test performed in 2018/2019 (a) and 2019/2020 (b) winter dormancy cycles. Fresh weight (in mg) of apical meristems were compared between natural (from the field) and forced conditions. Chilling hour accumulation (temperature < 7.2°C per hour) during winter 2018/19 (c) and 2019/20 (d).

3.2 Genome-wide gene expression analysis during dormancy

Based on the results mentioned above, four time-points from 07/01/2019 until 24/02/2019 and covering distinct dormancy phases were chosen for the RNA-seq experiment (Table 1). This experiment was done using GD as a referent genotype for apple studies. The first point corresponded to the endodormancy phase (called ENDO1), the second (ENDO2) and third (ECO1) to the transition endo-ecodormancy, and the last one to the ecodormancy phase (ECO2). Notably, significant changes in

field temperature were recorded between dates (Table 1). The RNA from bud samples of the four time-points was extracted and sequenced (see methods), resulting in more than 20 million reads per library (Table 1). The raw data were aligned to the reference genome (GDDH13) [49], and the differentially expressed genes (DEG) were identified through three different methods: edgeR likelihood, edgeR quasi-likelihood, and NOISeqBIO (Figure 3). The three methods yielded a similar number of DEG. However, the method quasi-likelihood was chosen for downstream analyses as it identified the greatest number of shared DEG with the other two methods (Figure 3a, b and c). Most of the DEGs were detected in the comparison between ENDO1 to ECO2 (Table 2). The global changes of gene expression detected using edgeR likelihood were visualized in a heat map (Figure 4), where it was observed that most of the genes are up-regulated from the ENDO2 onward (in red). Remarkably, the heat map evidences an inverted gene expression tendency that matches the transition between endodormancy (libraries ABC and DEF) to ecodormancy (libraries GHI and JKL).

Table 1-1 Samples, phenological stages, number of reads and coverage in genome reference of sequenced libraries

Time-point	date	Temp. in field	library	n° reads	coverage in GDDH13 (%)
ENDO1	07/01/2019	4-8°C	A	21301783	93,99
			B	21286437	94,18
			C	21188502	94,15
ENDO2	04/02/2019	4-6°C	D	20816727	94,01
			E	21355288	94,08
			F	21159342	93,96
ECO1	19/02/2019	6-7°C	G	21007186	93,93
			H	20948070	93,77
			I	20714606	93,66
ECO2	04/03/2019	10-15°C	J	21059577	94,08
			K	20986930	93,95
			L	20685328	94,10

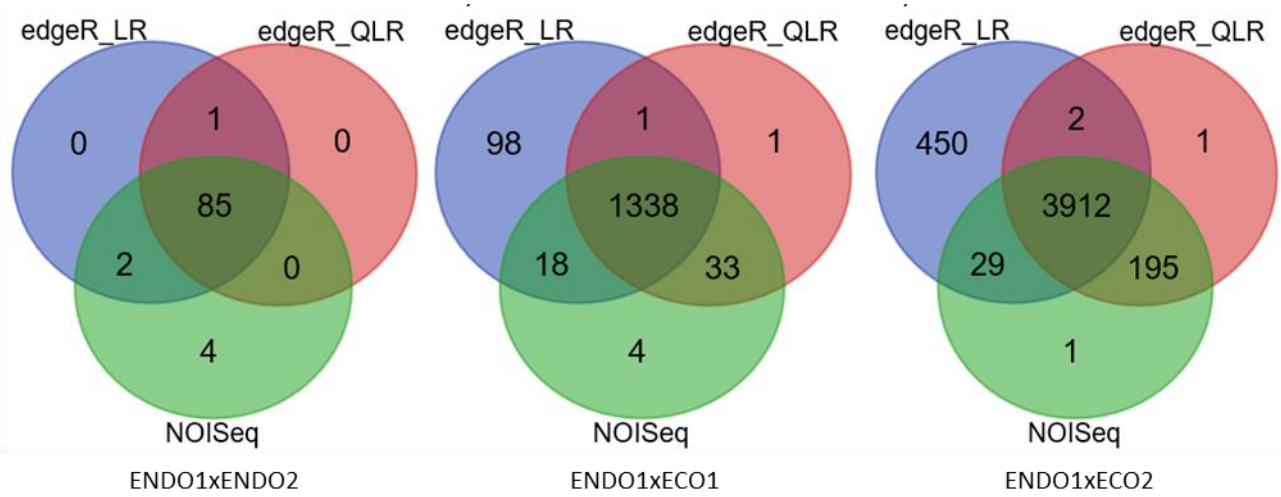


Figure 1-4. a) Comparisons among three methods to analyse DEG from RNA-Seq data. The three were calculated in R, with the methods edgeR likelihood (edgeR_LR, in purple), edgeR quasi-likelihood (edgeR_QLR, in red), and NOISeqBIO (NOISeq, in green), respectively. The three methods were applied in the comparisons of second, third and forth points against the first one, with log2FC > 2.

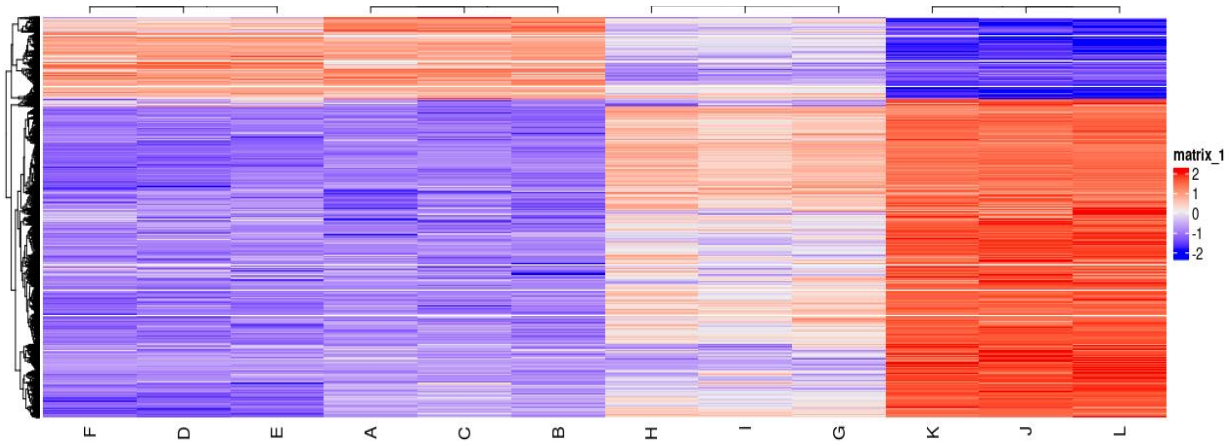


Figure 1-4.b) Heat map of expression profiles of genes differentially expressed (p-values<0,05) from RNA-Seq of Golden delicious buds during the dormancy cycle of 2018/2019. Blue and red colors indicate down and up-regulated genes, respectively.

Next, the DEG (FDR < 0.05 and log2FC > 2) were classified into expression clusters according to the pattern of expression. A dendrogram containing all DEG was built and used to select the minimum number of clusters representing the highest range of expression profiles (Figure 5a). In a preliminary approach, two options were selected, resulting in the classification of the DEG in six (Height value = 4.7) and four groups (Height value = 6.3), respectively (Figures 5b and 5c). These two groups differed just in two clusters that contained only a few genes. Therefore, the classification in four groups was selected as it contained the main observed patterns of expression in a minimum number of clusters (Figure 5c). Two of these clusters presented an increase in gene expression from endodormancy to ecodormancy (cluster1, from the ENDO2 point; cluster4, from the ECO1 point) and the other two

show an opposed behavior (cluster2, from the ECO1 point, cluster3 from the ENDO2 point). *FDR ≤ 0.05. total DEGs (^ upregulated/v downregulated)

Table 0-2 Number of DEG during the dormancy cycle 2018/2019 (the number of genes respectively up and down-regulated are indicated in brackets).

	Endo1 vs Endo1	Endo1 vs Eco1	Endo1 vs Eco2
log2FC ≥ 1*	700 (^268/v432)	4343 (^3226/v1117)	10091 (^6676/v3415)
log2FC ≥ 2*	115 (^8/v107)	1444 (^1264/v180)	4260 (^3388/v872)

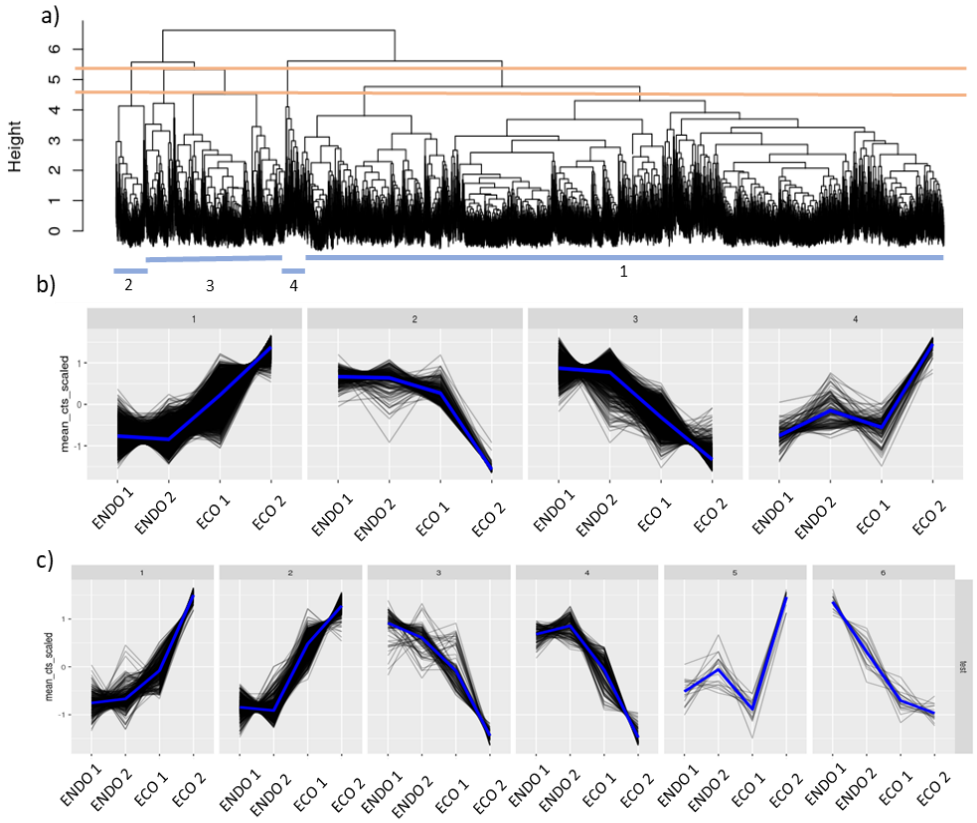


Figure 1-5 Dendogram and clusters of expression profiles of genes differentially expressed (p-value < 0.05) from RNA-Seq of Golden delicious buds during the dormancy cycle 2018/2019. Clustering was performed with the 'complete' method that cluster all the genes until a single cluster. Lines in orange represent the clustering selection shown in b) (upper with 4 groups) and c) (lower with 6 groups). Lines in blue represent the median value in each of the groups formed by clustering. c) Clustering with 6 groups, not used.

3.3 Functional classification of differentially expressed genes within the four clusters

The clustering shown above suggests that transition from endo- to eco-dormancy implies an important genetic reprogramming, as reflected by the significant transcriptomic changes observed between ENDO2 to ECO1/ECO2. In order to gain insights into the biological function underlying these changes, a Gene Ontology (GO) analysis was performed. This analysis resulted in the identification of predominant GO categories for each of the four clusters. From cluster1 is highlighted the functions related to organ development, response to hormone stimulus (mainly auxin, but also brassinosteroids and jasmonic acid), cell wall organization, DNA replication, mitosis, and meiosis, among others (Figure 6a). Cluster2 is enriched in DEG with functions related to response to temperature stimulus, cell redox homeostasis, and the starch metabolic process (Figure 6b). The functions enriched in cluster3 are mainly associated with response to hormone stimulus (abscisic acid, ethylene, and gibberellin), response to oxidative stress, regulation of transcription DNA-dependent, etc. (Figure 6c). The cluster4 presents weak enrichment, mainly in the gibberellin metabolic process (Figure 6d).

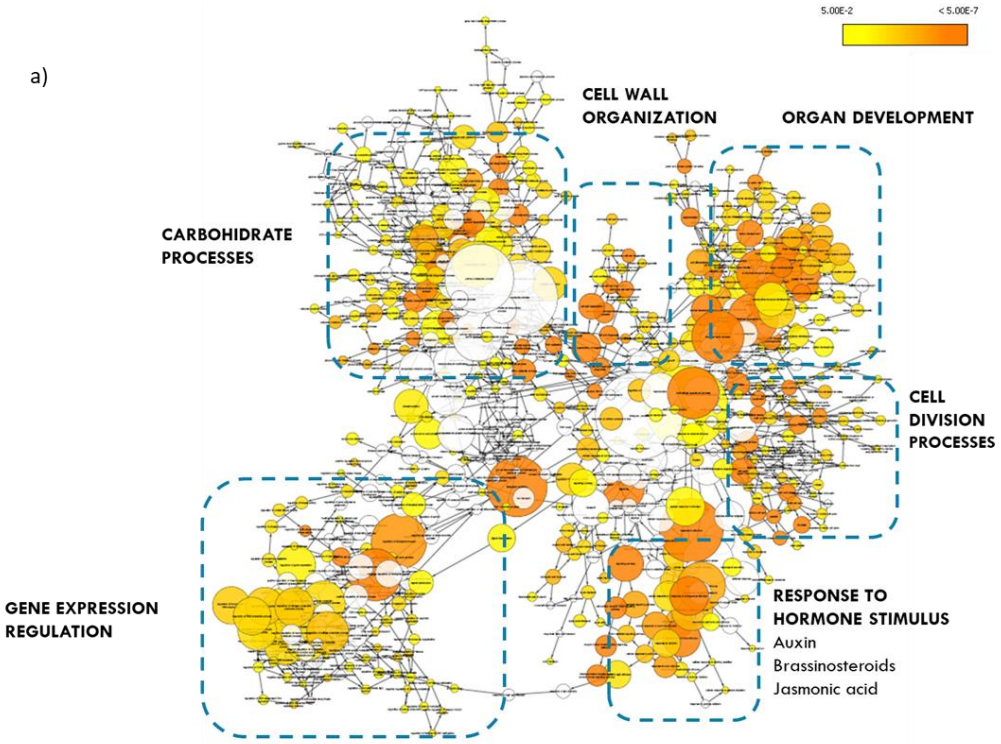
The DEGs in at least one comparison among the four time-points were also selected for the functional characterization by using a GO enrichment analysis. DEGs in the comparison ENDO2 vs. ENDO1 were enriched in functions associated with response to temperature stimulus (heat), fatty acid metabolic process, and secondary cell wall biogenesis, among others (Figure 7a). The comparison ECO1 vs. ENDO1 was enriched in DEG related to DNA replication, transcription transcription-dependent regulation, cell cycle regulation and cell division were mainly over-represented (Figure 7b). For the comparison of ECO2 vs. ENDO1, the most temporal distant points, the enriched GOs are related to the regulation of transcription, cell cycle process, response to light stimulus, response to hormones, and anatomical structure formation involved in morphogenesis, among others (Figure 7c)

Selection of candidate genes related to bud dormancy release

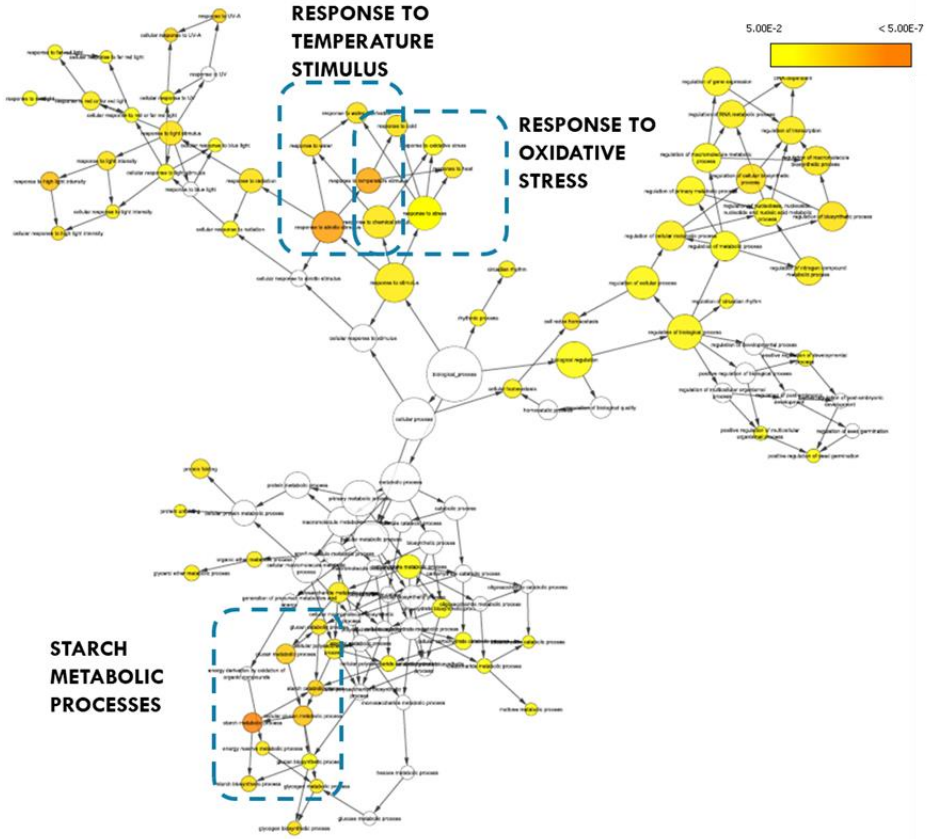
Several genes have been reported as potential components of gene regulatory networks controlling dormancy in apple and other Rosaceous plant species [3,24,60,61]. Moreover, many of these genes display specific expression profiles

during the dormancy cycle [3]. The expression profile of some of these genes (considered here as dormancy “marker genes”) was investigated in detail within our RNA-seq data. The “marker genes” were: *MdDAM1*, *MdDAM2*, *MdDAM4*, *MdDAMb*, *MdCBF4*, *MdEBB1*, *MdFLC*-like, *MdMAF2*, *MdSVPa* and *MdSVPb*. The expression pattern of these genes is presented in Figure 8. Marker genes were grouped into three categories depending on their expression profile. A group containing the expression patterns of *MdDAM1*, *MdDAM4*, *MdDAM2*, and *MdDAMb* showed a reduction of the mRNA levels toward ecodormancy. Characteristically, *MdDAM4* and *MdDAM1* had their peak of expression during endodormancy (Figure 8a). The second group of genes, *MdFLC*-like, *MdEBB1*, and *MdMAF2*, displayed an increase in gene expression from the end of endodormancy until the bud break (Figure 8b), and a third group containing *MdSVPa* and *MdSVPb* presented a stable gene expression during the dormancy-cycle (Figure 8c). The expression profiles shown by the “marker genes” in our RNA-seq experiment were consistent with previous reports [3,60,62].

a)


5.00E-2
< 5.00E-7
CELL WALL ORGANIZATION
ORGAN DEVELOPMENT
CARBOHIDRATE PROCESSES
CELL DIVISION PROCESSES
GENE EXPRESSION REGULATION
RESPONSE TO HORMONE STIMULUS
Auxin
Brassinosteroids
Jasmonic acid

b)


RESPONSE TO TEMPERATURE STIMULUS
RESPONSE TO OXIDATIVE STRESS
5.00E-2
< 5.00E-7
STARCH METABOLIC PROCESSES

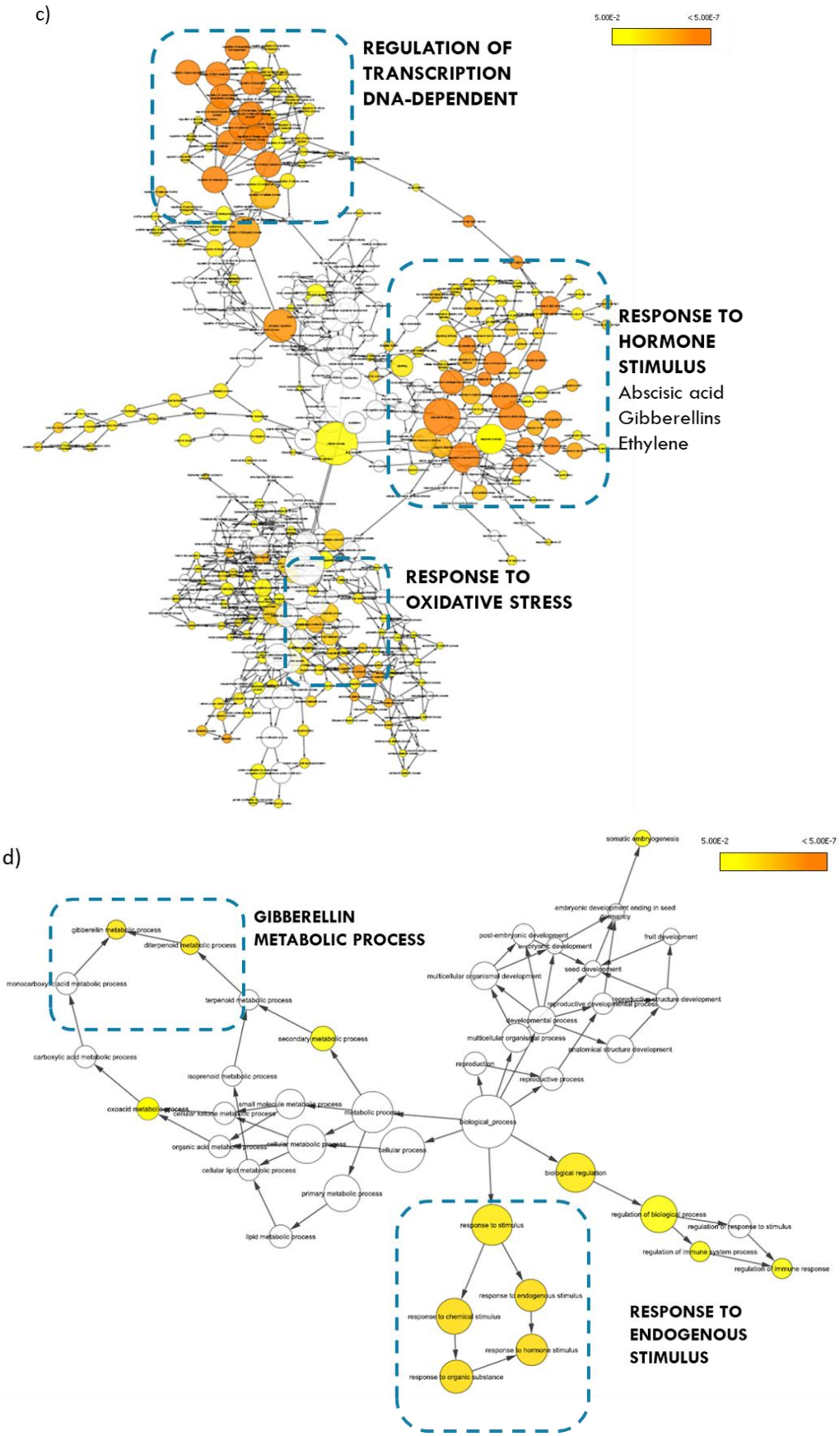
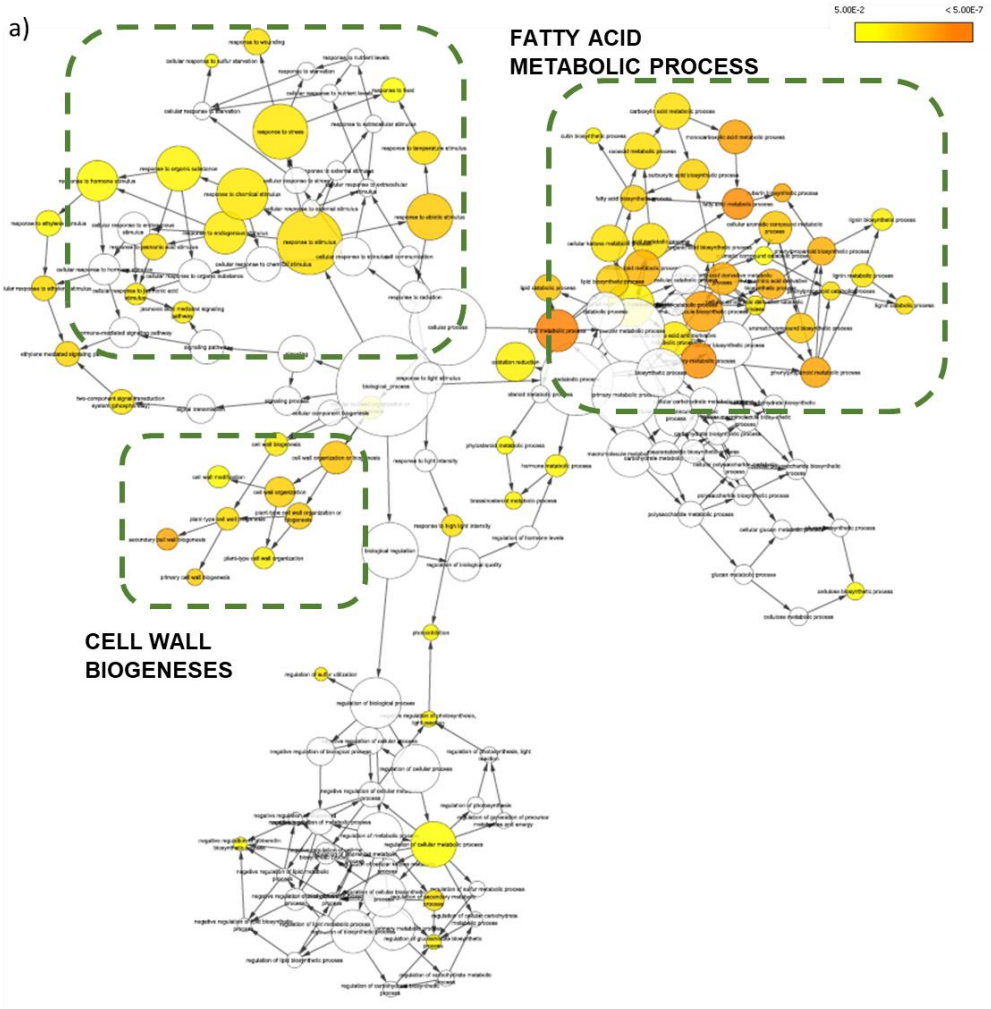
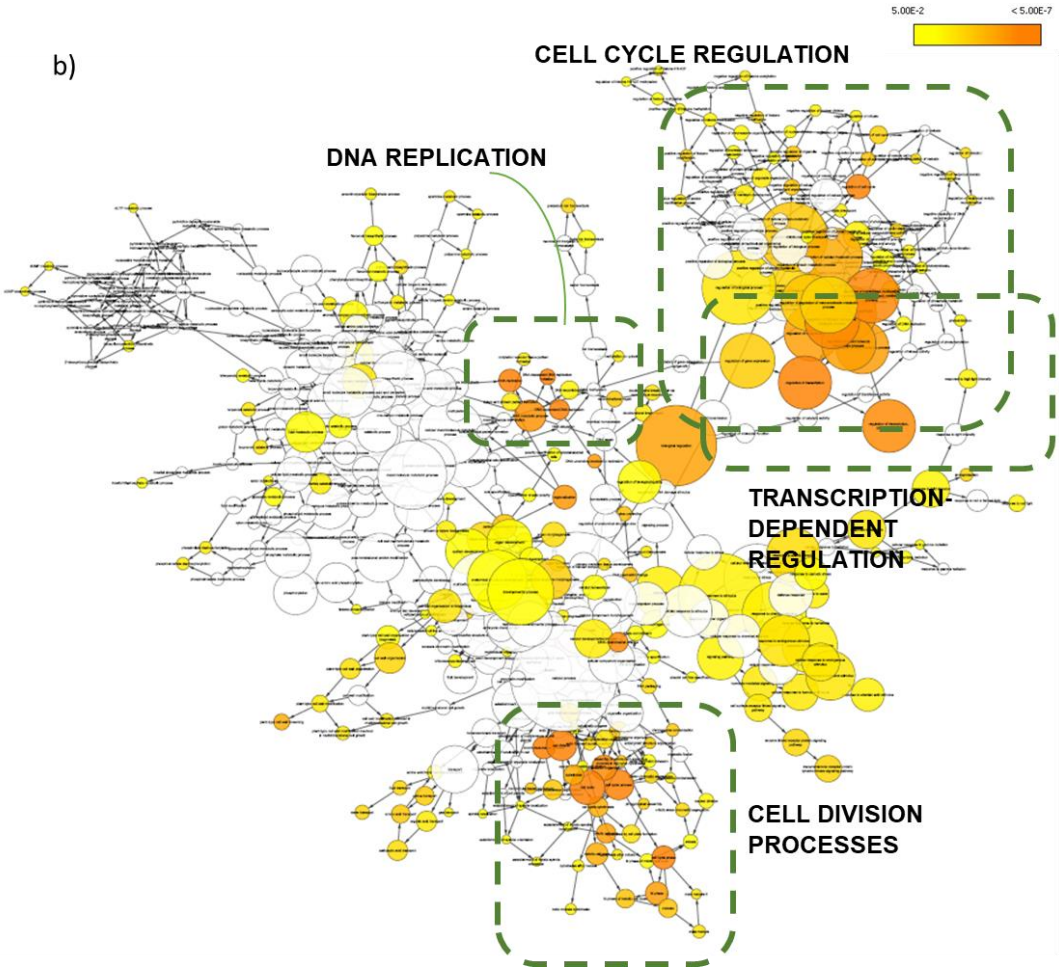


Figure 1-6 Gene Ontology (GO) of the genes differentially expressed belonging to each of the four clusters previously selected a) cluster1 b) cluster2 c) cluster3 d) cluster4


a)
5.00E-2
< 5.00E-7
FATTY ACID
METABOLIC PROCESS
CELL WALL
BIOGENESES


b)
5.00E-2
< 5.00E-7
CELL CYCLE REGULATION
DNA REPLICATION
TRANSCRIPTION-DEPENDENT REGULATION
CELL DIVISION PROCESSES

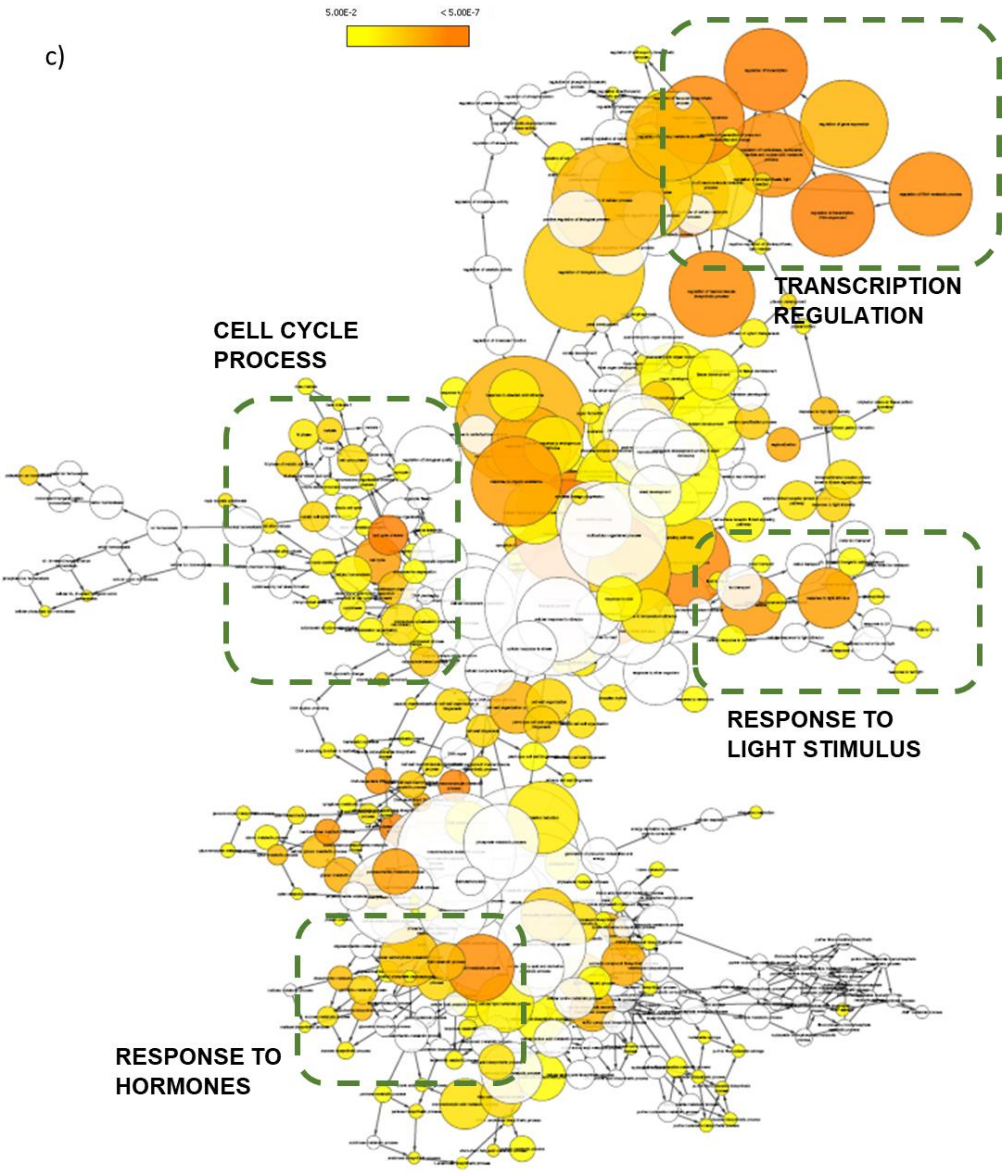


Figure 1-7 Gene Ontology of genes differentially expressed compared between: a) ENDO1xENDO2; b) ENDO1xECO1 and c)ENDO1xECO2

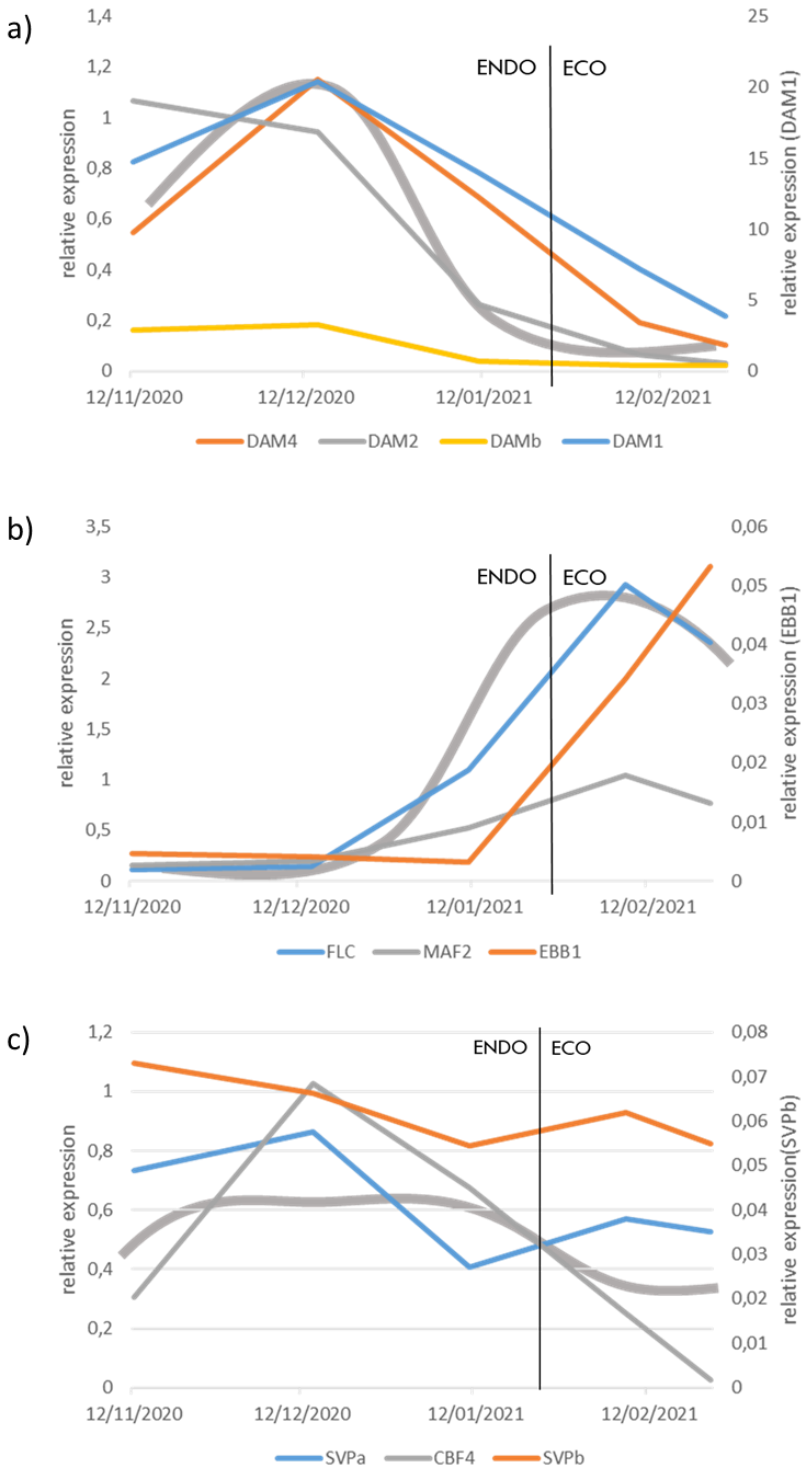


Figure 1-8 Representation of patterns of expression profiles of the genes already known as related to bud dormancy (marker genes)

The expression analysis of dormancy “marker genes” confirmed their potential function during dormancy. However, our RNA-seq data suggest that the dormancy cycle in apple is controlled by intricate networks formed by hundreds of genes different from those already pointed out in previous studies. In order to identify potential novel dormancy regulators, the list of DEG was screened for genes that, according to the literature, and their predicted GO function might play roles during the dormancy cycle. In this regard, the main criteria chosen are (1) a molecular function related to transcriptional regulation (e.g., transcription factor, DNA binding, etc.) and (2) the genes that show a GO enrichment for a biological function relevant during the dormancy cycle, i.e., drought and cold tolerance, flowering time control, hormonal signaling, response to warm temperatures and redox homeostasis. A total of 10 genes were selected for gene quantification assays (Table 3). In order to further characterize the possible role of these genes in the control of dormancy, the expression levels were quantified in the GD and PL buds collected in 2018/19 and 2019/20 winters. For the selected genes, only *MdHB22 (MdHOMEOBOX PROTEIN 22*) and *MdANT* (*MdAINTEGUMENTA*) presented statistically different expression levels between GD and PL. Indeed, the expression of *MdHB22* and *MdANT* was higher in PL compared to Golden at the end of ecodormancy in samples from 2019/2020 (Figure 9). However, the expression levels of neither the other selected genes (Table 3) nor marker genes were significantly different between cultivars (Annex 4).

Table 1-3 List of selected candidate genes to be tested

Gene (GDDH13)	Code	Function based in TAIR	(TAIR)
MD14G1241400	HB22 - homeobox protein 22	Dormancy related	AT4G24660
MD15G1064600	ANT - Integrase-type DNA-binding superfamily protein	flowering/AP2 related	AT4G37750
MD00G1036300	HB-7 - homeobox 7	Cold (ABA/drought related)	AT2G46680
MD04G1067900	DREB1A/CBF3 - dehydration response element B1A	Cold (ABA)	AT4G25480
MD08G1035400	BZR1 - Brassinosteroid signalling positive regulator (BZR1) family protein	flowering (ABA and brassinosteroids)	AT1G75080
MD08G1241900	BAM2 - Leucine-rich receptor-like protein kinase family protein	Dormancy/flowering	AT3G49670
MD09G1264800	RGA-like 2	Dormancy (gibberellins, possible role in ROS)	AT3G03450
MD10G1041100	SEP4/ AGL3 - K-box region and MADS-box transcription factor family protein	Cold (flowering)	AT2G03710
MD14G1086100	ARR9 - response regulator 9	cytokinin/circadian clock	AT3G57040
MD15G1075800	SLY1 - F-box family protein	Dormancy (gibberellins)	AT4G24210

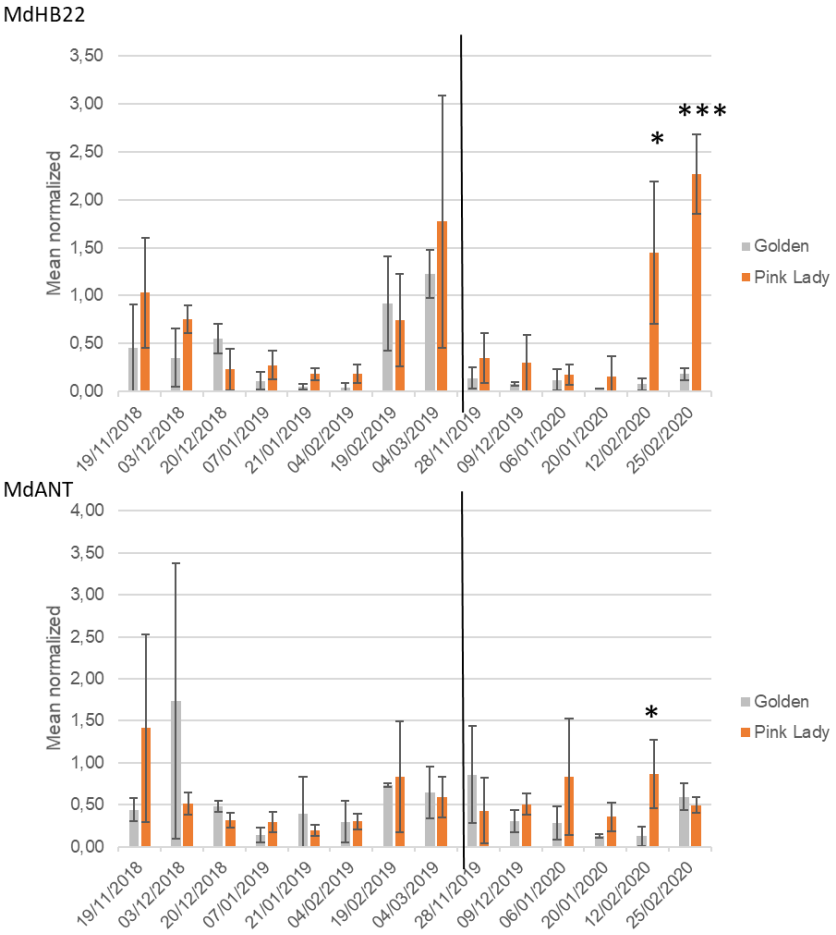


*Figure 1-9 Gene expression profiles of MdHB22 and MdANT in Golden delicious (gray bars) and Pink Lady (orange bars) cultivars, during two dormancy cycles. * p<0,05 *** p<0,001*

3.4 Detection and quantification of differentially alternatively spliced isoforms (DASI) during dormancy

In order to get insights into the posttranscriptional control of the dormancy cycle in apple trees, the RNA-seq data was re-analyzed to identify and quantify DASIs during dormancy. The bioinformatics' Tuxedo pipeline was used for the genome-wide identification and quantification of gene isoforms. By applying this pipeline to the RNA-seq data and using the GDDH13 reference genome [49], a total of 48,936 isoforms from 28,991 genes were identified. The other 16,125 genes were identified as having a single isoform and were thus not considered in further AS analyses.

The genes used as a reference for the dormancy (marker genes) were first analyzed to identify the number of isoforms, the profile of expression, and isoform

composition. This first analysis revealed unexpected results related to the isoform prediction done by the Tuxedo pipeline. For example, for the gene *MdDAM1* (MD15G1384500), three of four detected isoforms were longer than the annotated gene, covering even another adjacent gene, *MdDAMb* (MD15G1384600) (Figure 10a). The *MdDAM1* exon-intron structure predicted by Tuxedo clearly differed from the one shown at the NCBI Genome Data Viewer (GDV) (https://www.ncbi.nlm.nih.gov/genome/gdv/). This was not an isolated case and despite the pipeline being re-run several times, such abnormalities were found recurrently. Unfortunately, our efforts to identify the problem during the pipeline execution were vain, and nowadays, we do not have a solution to obtain optimal results by using Tuxedo. Nevertheless, we keep working on the adaptation of the pipeline to the apple genome and the RNA-seq data we have. Meanwhile, we focused on a manual search of AS of the dormancy-related MADS genes by combining the information from the NCBI GDV and our RNAseq data. For this, we looked for differences in the lines, as illustrated in Figures 11-13, as they represent the number of reads supporting a particular intro-exon configuration.

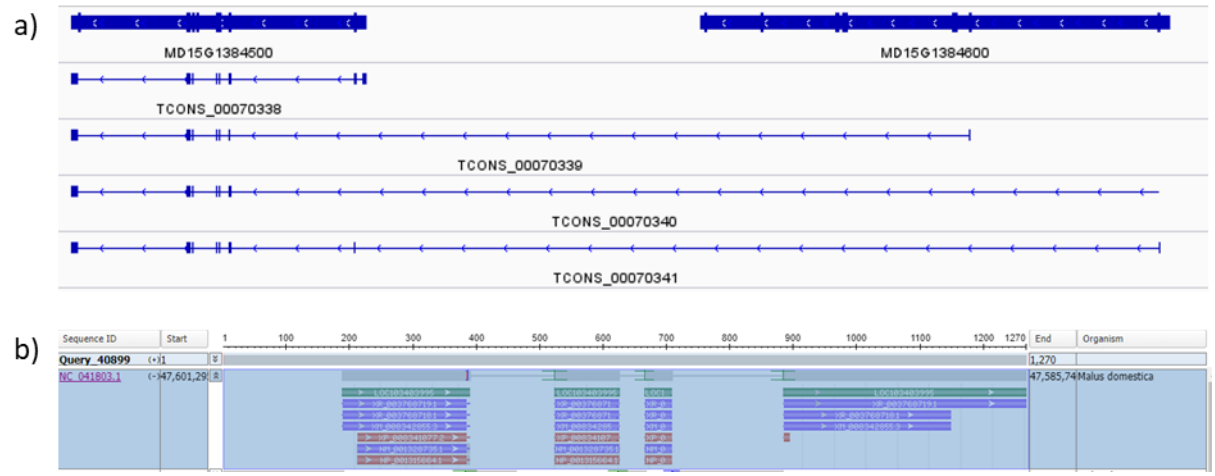


Figure 1-10 a) Example of isoforms for the gene MdDAM1 (MD15G1384500) found by Tuxedo, 'TCONS' are the indicator of isoform found, being the blue boxes the exons and the arrow indication the transcription reading sense. b) Example of isoforms for LOC103403995 in IRHS genome, the correlate of MdDAM1 (MD15G1384500).

From the ten investigated dormancy-marker genes (*MdDAM1, MdDAM2, MdDAM4, MdDAMb, MdCBF4, MdEBB1, MdFLC-like, MdMAF2, MdSVPa* and *MdSVPb*), three of them presented possible AS: MdMAF2 (MD05G1037100), *MdFLC*-like (MD09G1009100) and *MdSVPa* (MD01G1038600). In the case of *MdMAF2,* a

potential DASI was identified. The small extra exon added is illustrated as the point of convergence between two arched lines from the annotated exon 1 and exon 2 (Figure 11). This possible increase of mRNA from the configuration that includes an extra exon is observed during ECO1 and ECO2. In the next step, the two configurations of *MdMAF2* were quantified by RT-qPCR using specific primers. This suggested that only the annotated gene version changes its expression levels during dormancy (i.e., increases from ENDO1,2 to ECO1,2). Similarly, the potential extra exon of *MdFLC,* between the first and the second exons (Figure 12a), did not show differences in mRNA expression when compared to the annotated gene version (Figure 12b).

For *MdSVPa*, a configuration including a longer exon 8 was identified. The short and long isoforms of *MdSVPa* are shown in Figure 13a. The representation of the RNA-seq reads alignment on the GDDH13 apple genome using the IGV visualization tool suggests an increase of the MdSVPa long-exon 8 expression during ecodormancy compared to endodormancy (Figure 13b). Using primers that specifically detect that the *MdSVPa* exon 8 would be progressively more expressed according to the dormancy progression (Figure 13b). Still, we saw a U shape expression in our gene expression quantifications, starting in middle expression, getting lower just before the endodormancy release, and increased expression in ecodormancy. We measured the expression of exons 7 and 8, and remarkably the exon 7 was higher expressed in the point just before the endodormancy release, being the other point not significantly different

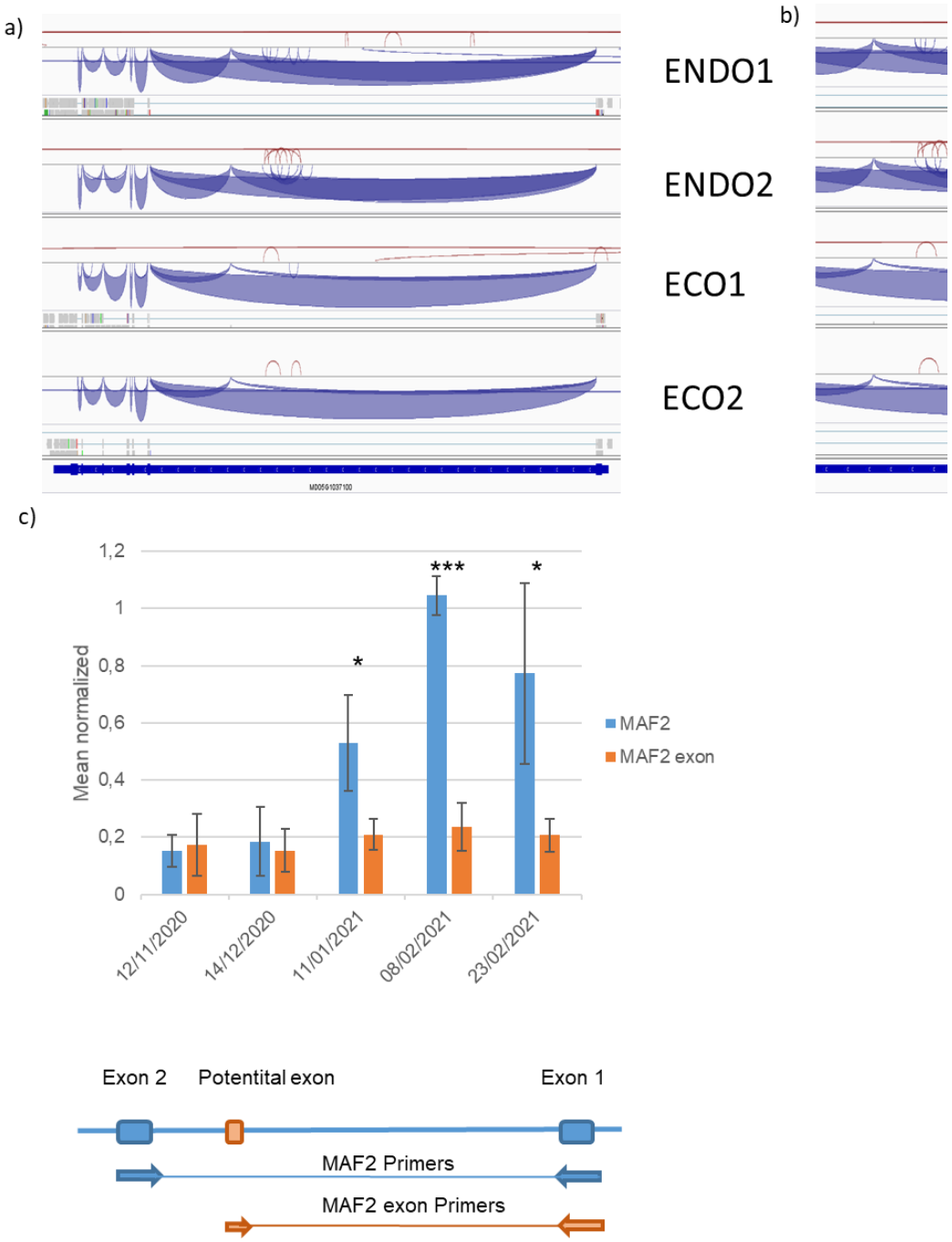


Figure 1-11a) Alignments of RNA-seq data to the reference genome for the 4 stages sequenced, focusing in MAF2 gene (MD05G1037100) b) Detail of the possible exon, situated between the annotated exon 1 and exon 2. c) Quantification of isoforms expression with primers anchoring in the main gene (MAF2) or in the putative exon (MAF2 exon). Map of primers is shown in detail at the bottom of the Figure.

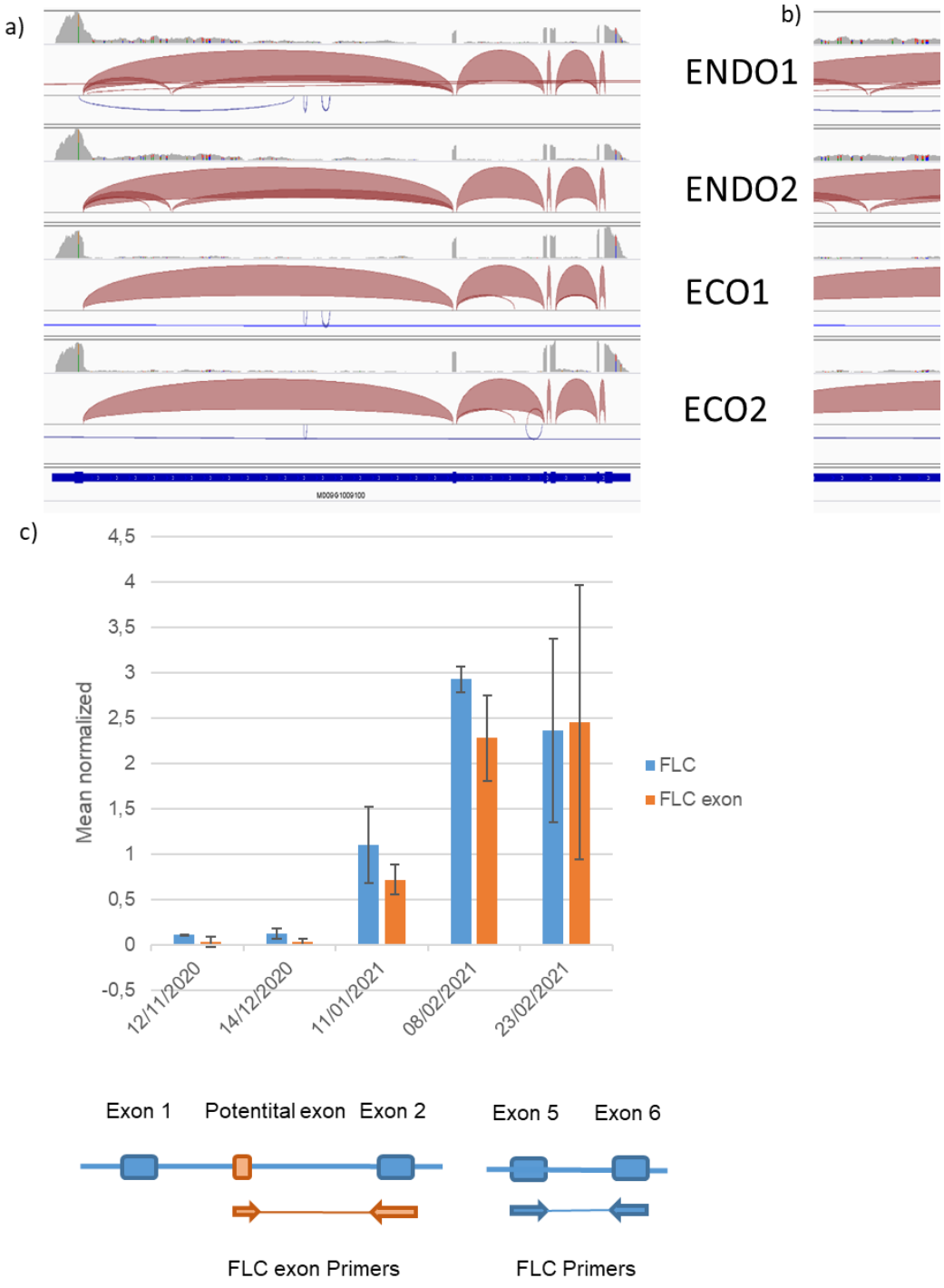


Figure 1-12 a) Alignments of RNA-seq data to the reference genome for the 4 stages sequenced, focusing in FLC-like gene (MD09G1009100) b) Detail of the possible exon, situated between the official exon 1 and exon 2. c) Quantification of isoforms expression with primers anchoring in the main gene (FLC) or in the putative exon (FLC exon). Map of primers is shown in detail at the bottom of the Figure.

For *MdSVPa*, an isoform that presents an additional 8th exon was identified instead of the seven-exons gene structure as the reference genome [49]. The short and long isoforms of *MdSVPa* are shown in Figure 13a. The representation of the RNA-seq reads alignment on the GDDH13 apple genome using the IGV visualization tool suggests an increase of the *MdSVPa* long-exon 8 expression during ecodormancy compared to endodormancy (Figure 13b). Using primers that specifically detect that the MdSVPa exon 8 would be progressively more expressed according to the dormancy progression (Figure 13c). Still, we saw a U shape expression in our gene expression quantifications, starting in middle expression, getting lower just before the endodormancy release, and increased expression in ecodormancy. We measured the expression of exons 7 and 8, and remarkably the exon 7 is higher expressed in the point just before the endodormancy release, being the other point not significantly different.

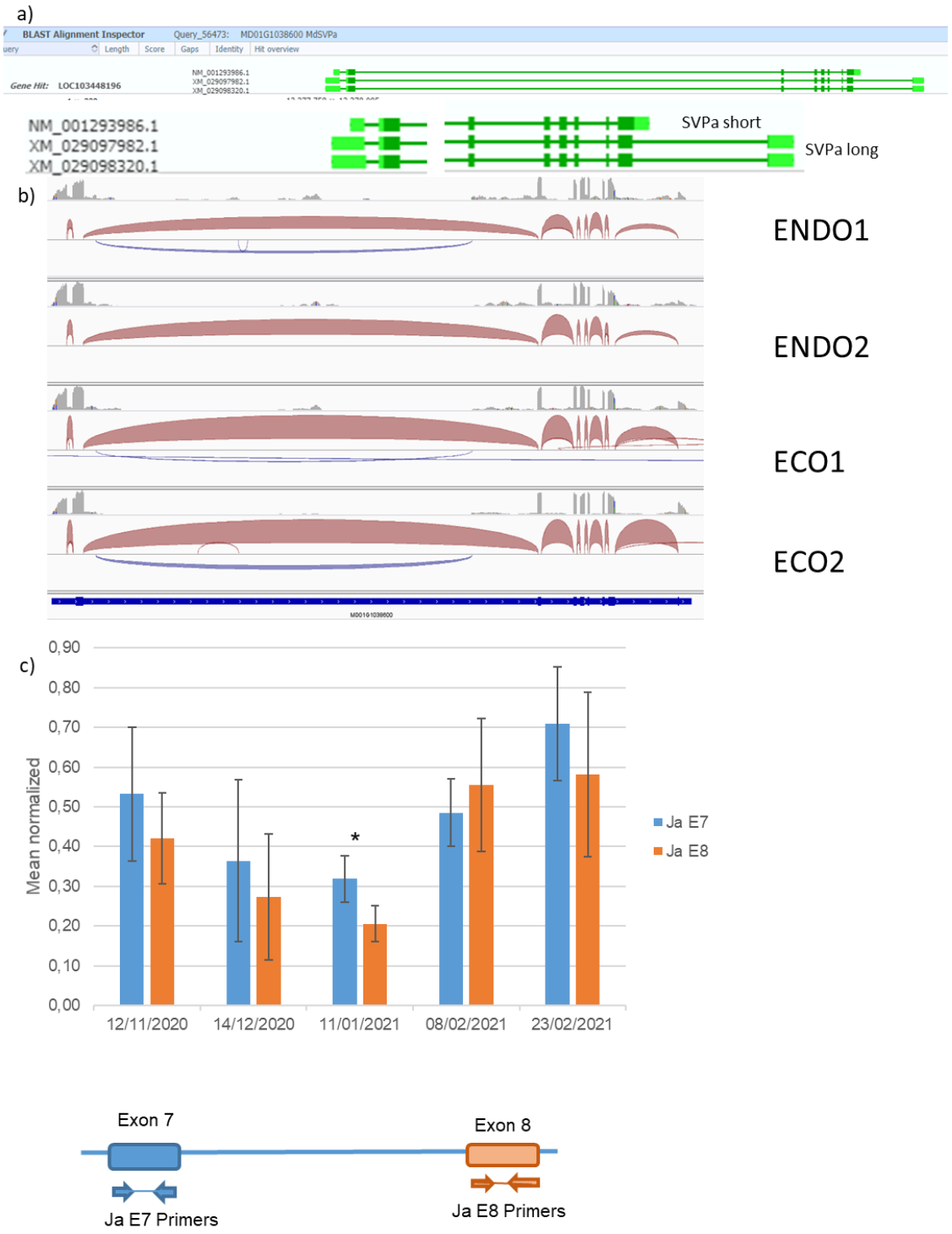


Figure 1-13 a) Isoforms identified by the IRHS genome, showing 3 possible isoforms. b) Alignments of RNA-seq data to the reference genome for the 4 stages sequenced, focusing in SVPa gene (MD01G1038600) c) Quantification of isoforms expression with primers anchoring in the main gene (Ja E7) or in the putative exon (Ja E8). Map of primers in gene is shown in detail at the bottom of the Figure.

4 DISCUSSION

4.1 Diversity of CR and bud break timing between two apple cultivars denote genetic control of the dormancy-cycle

Bud dormancy is an adaptive mechanism that allows plants to face the hazards of the winter season and to reactivate growth in the spring by having different responses to chilling and warm temperatures. The aim of this project was to understand the genetic modulation that happens exactly in the transition from endodormancy to ecodormancy. For this reason, specific phenological studies were performed. The Tabuenca and bud burst cutting tests were used to determine the phenological state. We used two distinct cultivars, GD and PL, which present different CR, HR and flowering dates. PL switched from endo- to ecodormancy at 450CH and 400CH in 2018/19 and 2019/20, respectively (Figure 3 a,b). For GD, it happened at 960CH and 700CH (Figure 3 a,b). Both CR match what is found in literature and recommendations from breeding programs about the CR of cultivars in France and also South Africa [43,63]. This shows that despite the year or location, the cultivars lean towards their defined CR, illustrating the importance of the genetic background. Despite the huge difference in CR, the flowering dates in the field are not that diverse, being 12 days of difference in flowering in the field between cultivars in 2019 and 18 days in 2020, while the endodormancy release occurred at 26 days and 29 days in 2019 and 2020, respectively. The differences between GD and PL are not only in the CR but also in flowering dates. After the endodormancy release, PL achieved flowering with 238 heating hours (HH, temperature > 15°C) in 2019 and 151 HH in 2020. For GD, it was 275 HH and 259 HH in 2019 and 2020, respectively. The differences in HR seem minimal between the cultivars (close to 100 HH), despite the significant difference in CR (~400 CH for PL and ~800 CH for GD). As the ecodormancy is the environmental repression of growth, instead of the endodormancy in which the internal genetic factors are the blockers of growth [1], we can indicate that genetic control is evident in the endodormancy, but in ecodormancy it is less evident, especially in winters in which enough chilling is experienced. Differences were observed between cultivars in warm conditions, but in our case, enough chilling allowed the proper acquisition of CR by both cultivars [64].

The phenological study of the two cultivars showed the genetic influence in the endodormancy, according to their CR, and that the HR is not that diverse between them.

4.2 The transition from endo-to ecodormancy implies a large meristem transcriptomic reprogramming

The RNA-Seq performance was satisfactory with good quality according to the FASTQC files individually and well the alignments to the genome (around 94% in the average of all samples) as well as the choice of the most suitable method of analysis, the EdgrR QLR. The process of characterization of genetic influence in phenological observation previously discussed can be based on DEGs (FDR < 0.05 and log2FC > 1 or 2). We have chosen four time-points very close, and that cover the exact transition from endo- to ecodormancy. Our choice for those points was based on the focus on understanding the genetic changes from the endodormant to the ecodormant phase and, with that, potential genes controlling this switch. Table 2 is shown the DEGs with log2FC ≥ 1 and log2FC ≥ 2 (FDR ≤ 0.05) in the comparison of ENDO1 against the other time-points, ENDO2, ECO1 and ECO2. With the progression of time, the comparisons present a larger quantity of DEGs than the previous comparison, from 700 DEGs in ENDO1 *vs.* ENDO2 to 10091 DEGs in ENDO1 *vs.* ECO2 (log2FC ≥ 1; FDR ≤ 0.05) in a genome of 52741 genes [49]. Being more restrictive (log2FC ≥ 2; FDR ≤ 0.05), we obtain fewer DEGs as 115 for ENDO1 *vs.* ENDO2, 1444 for ENDO1 *vs.* ECO1 and 4260 for ENDO1 *vs.* ECO2. The DEGs are remarkable in ENDO1 *vs.* ECO1 but very high in ENDO1 *vs.* ECO when almost 10% of genes are log2FC ≥ 2 differentially expressed. Such genetic reprogramming is quite common in meristems phase switching, such as vegetative to flowering in *A. thaliana* or bud dormant to non-dormant in other *Rosaceae* fruit trees [21,23,65,66]. More interestingly, despite just 8 of 115 DEGs from ENDO1 *vs.* ENDO2 were up-regulated, 1264 of 1444 and 3388 of 4260 DEGs were up-regulated in ENDO1 *vs.* ECO1 and ENDO1 *vs.* ECO2, respectively. This shows not only the great reprogramming in the meristem but also the activation of transcription of these genes, suggesting a process of 'awakening' from the endodormant state.

Focused on the most intense genetic variation, we decided to use only DEGs having FDR < 0.05 and log2FC > 2 for their clustering according to their pattern of

expression identified in the RNA-Seq. We could classify the genes into four groups according to their pattern of expression. Interestingly the great majority of the DEGs are in cluster1, which presents up-regulation from ENDO2 onward. The second biggest one is cluster3, which is down-regulated in opposition to cluster1, from ENDO2 onward. This shows that the biggest differences appear from this point to the next ones, from the endodormancy to the ecodormancy state, as we previously discussed [21,67]. This also demonstrates how the Tabuenca test properly selected the endodormancy release and correct time-point selection for the sequencing. This behavior is expected for genes that have an impact on endodormancy release, either by the down- or up-regulation, once the phenological effect (endodormancy release visualized by Tabuenca and/or Bud break test) should happen after the genetic transition.

4.3 Several biological processes are affected during the endodormancy release

After the proper choice of the dates, which were performed with a high temporal resolution, the transcriptomic profiling allowed us to better understand the biological and molecular processes undergoing during endodormancy release and the beginning of ecodormancy. Following the identification of DEGs and their clustering according to their pattern of expression, the identification of biological and molecular functions was necessary. Starting with identifying the enriched GOs, the genes in the cluster1 present pathways related to organ development, cell wall organization, DNA replication, mitosis and meiosis, which is related to floral organ preparation for bud burst and awakening of metabolism to the fast growth that is coming, as well for the gamete formation and final steps of flowering organs definition (Figure 7a). This shows that most of the biological processes associated with the function of the DEGs in cluster1 are related to growth and preparation for the endodormancy release. This agrees with the previous observations made in cherry, with higher transcriptional differences between endo- to eco- than inside stages [18]. With antagonistic behavior (i.e., down-regulated from ENDO2 to ECO1/2), cluster3 presents enrichment in GOs related to the regulation of transcription (DNA-dependent), mainly represented by transcriptional repression. This could indicate a strong release of transcription occurring during the endo- to eco-dormancy transition. Also, this cluster was enriched in GOs for response to hormone stimulus (ABA and GA), which are known for their participation in dormancy (ABA). This shows that the genes related to ABA and the repressor machinery of transcription

are slowly down-regulated towards ecodormancy and bud break in buds of the apple tree. These finds match the finds of previous RNA-Seq of dormant apple buds releasing dormancy [23]. Cluster2 (down-regulated from endo- to ecodormancy) has highlighted the functions related to response to temperature stimulus, cell redox homeostasis, and the starch metabolic process, an indication of the high sensibility to temperature in endodormancy (ENDO1 and ENDO2), possibly associated to the response to oxidative stress as observed in [68]. The cluster4 (up-regulated from ECO1 to ECO2, mostly) presents weak enrichment in GO categories. Mainly the gibberellin metabolic process was enriched in this cluster, which can be related to the growth and flowering development expected for the flowering process, related to the gibberellin flowering pathway in *A. thaliana* [69], as well as the regulation of dormancy observed in peach and grapevine [70,71]

Finally, the GO enrichment analysis allows us to conclude that the transition from endo- to eco-dormancy (ENDO2xECO1) presents a release of the epigenetics control and permission for activation of the transcription machinery, which results in a shift to growth and flowering, including processes related to mitosis, meiosis and flower organ development. In addition, this dormancy-phase transition is characterized by enrichment in GO categories related to redox signaling. Just after, already in ecodormancy (ECO1xECO2), we can see that the most active pathways are related to growth. During this period, there is not a specific enrichment for response to oxidative stimuli, suggesting a lower influence of redox signaling. Thus, these results suggest that the end of endodormancy and the endodormancy release is highly related to oxidative stress pathways. Previous reports of oxidative activity of seed dormancy release with hormonal pathways are similar to our finds. Potentially seed dormancy is similar to bud dormancy at that point, as already suggested [72–75]. A burst of redox response at the endo- to eco-dormancy transition might be contributed by the simultaneous epigenetic release of the transcriptional machinery.

Focusing on the comparison during endodormancy release, the GOs enriched in the ENDO1xECO1 are mostly related to the regulation of transcription, DNA replication, cell division and cell wall loosening. This is another demonstration of the removal of a repression system that allows the reactivation of general transcription and growth. In the case of ENDO1xECO2, GOs are highly represented for general hormone responses and for transcriptional regulation and epigenetic control, which further

reaffirms the great metabolic shift of this transition. Those findings match the ones of RNA sequencing during endodormancy release in apple [76,77], sweet cherry [18,66,67] and peach [78].

4.4 Genes related to dormancy might not be involved in the phenological differences between GD and PL

The bud dormancy control has been classically related to the expression of dormancy-related genes such as the *DAMs* and the *SVP*-like genes [21,60,79]. DAM genes are sequentially expressed during the dormancy cycle in apple and other fruit trees in a way that their expression profile correlates with the acquisition of CR [60] [3]. We studied the four major apple *DAMs* (*1*, *2*, *4* and *b*) and the two *SVP*-like genes (a and b), in addition to *CBF4*, *EBB1*, *FLC*-like and *MAF2,* which are commonly associated with dormancy phases and progression [8,79,80].

The expression pattern of these genes can be classified into a group representing genes showing high expression in the endodormancy until endodormancy release and the second group of genes being up-regulated after endodormancy release during ecodormancy until flowering. These groups of genes were chosen to act as markers and to permit the proper placement of the new studied genes. The aim of this action is to place the candidate genes in the proper place within the pathway. The *DAM* genes (*1-4* and *b*) present the expected downregulation just before the endodormancy release, while *MAF2*, *FLC*-like and *EBB1* present upregulation after endodormancy release. Interesting to point out that among these genes, only *DAM1*, *DAM2*, *DAM4*, *CBF4* and *EBB1* presented quantifiable reads in the RNA-Seq (annex 4). These genes were quantified in our samples from the two dormancy cycles in GD also in PL. The quantification of gene expression presented a pattern that matches the ones already observed [21,60]. In particular, the *DAMs* are localized in cluster3, while the up-regulated genes (*FLC*-like, *EBB1* and *MAF2*) are in cluster1. As many other genes were in those two major clusters, potentially more of them could be associated with or participating in the dormancy control, and by that, more studies are necessary. Important to highlight that no differences were observed between the GD and PL cultivars in relation to the quantified dormancy marker genes. The forcing tests demonstrated that PL presents endodormancy release approximately three weeks before GD, while the flowering in the field is just two weeks apart in 2018/19 winter.

This matches the historical data that shows that PL presents a shorter endodormancy by smaller CR but a longer ecodormancy [43], possibly based on a higher HR. This is not itself an indicator of the absence of genetic divergences between the cultivars; once the bud break and Tabuena tests indicate a distinguished phenological state. This mismatch between the phenology and gene expression profile of known regulators of dormancy suggests that these genes, so-called master dormancy regulators, are not the exclusive regulators of this process. Interestingly the *DAM* genes were first described in peach in the classical *evergreen* natural mutant that does not enter dormancy [10]. Also, trees artificially impaired in *DAM1* function in apple do not enter into dormancy. Potentially, *DAM1* is a dormancy inductor, but it cannot be ruled out that other genes different from *DAMs* could be responsible for the early bud break of PL, which seems to be related to a weaker endodormancy phase.

Set of Genes chosen for studies in distinct apple cultivars

The disconnection between the phenological differences between GD and PL cultivars and the expression of *DAMs* led us to study more genes found in the RNA-Seq. For that question, ten new genes were tested between both cultivars during the two distinct dormancy cycles. The chosen genes have GOs related to dormancy and temperature responsiveness as well as flowering induction. Their pattern of expression is mostly up-regulated in endodormancy release, indicating their possible activity in bud burst induction, in opposition to the other three chosen genes that can have relation to dormancy maintenance (Annex I, II). No differences were observed between the cultivars for eight of the ten genes, which indicates that they may not participate in the early phenological dormancy released earlier in PL. The two genes with statistical differences between cultivars were the *AINTEGUMENTA* (*MdANT)* (MD15G1064600) and *HOMEO-BOX 22* (*MdHB22)* (MD14G1241400).

MdHB22 is part of a well-described zinc-finger homeobox family. *MdHB22* is expressed predominantly in the floral tissue of *A. thaliana*, indicating a potential regulatory role during floral development [81]. Interestingly, *AtHB22* is highly expressed in older flowers and seeds [81], which matches the bud dormancy process and the late floral development in the apple tree, with differentiation of ovules and pollen sacs occurring at the end of the winter [82]. In pear, it is shown that ABA activates the transcription of *HB22*, which in turn activates *PpDAM1*, in an independent but parallel pathway for *DAM1* activation by CBFs [83,84]. Here, we have observed that *MdHB22 is* induced after the endodormancy release in both GD and PL cultivars

in particularly cold conditions (winter 2018/19). Specifically, for PL, there is an anticipated gene expression activation of *MdHB22* in PL in warmer conditions, consistent with the endodormancy release in that year (Feb/2020 was ~4°C higher than Feb/2019 when both cultivars presented the same pattern expression). This pattern led us to think that *MdHB22* is a dormancy repressor in warm conditions during ecodormancy, leading to bud break. As a TF, HB22 tends to form transcriptional complexes. Indeed, the opposite behavior in activating or repressing target genes could be explained by the type of dimer formed by HB22 with itself (homodimers) or other TFs (heterodimers). This peculiarity has already been described by members of the same TF family [85,86].

The *A. thaliana* protein ANT is a TF belonging to the AP2 family [87–89]. The ANT consensus site shows some similarities to the C-repeat/DRE elements. The ANT is a crucial factor in controlling the initiation of integument development. This gene is responsible for: 1) regulating organ initiation and size by potential proliferative maintenance of organ primordia [87,88,90]; 2) regulation of floral identity and organ polarity, together with *YABBY* genes [91]. *ANT* presents other roles during ovule initiation and development. Mutations in *ANT* result in a failure of the ovule to initiate integument formation [87,88]. There is evidence that the brassinosteroids, through the *BZR1* gene, could regulate de ANT activity [92], but no correlation between *BZR1* and *ANT* expression was found in our analysis. *ANT* appears to be regulated by ABA, with increased expression under drought conditions and ABA treatment [93]. Also, it was shown that ANT negatively regulates *COLD-REGULATED15A* (*COR15a*). *COR15A* and other *COR* genes respond to environmental stresses, especially drought, cold, and dehydration, usually associated with ABA signaling [94,95]. This indicates the connection of *ANT* in regulating flowering and coordinating environmental responses, particularly to cold.

MdHB22 and *MdANT* present differential expression between the GD and PL cultivars (with diverse CR and bud break dates) and might indicate their involvement in the final stages of the bud dormancy process. It is interesting that no remarkable differences in the expression of these two genes were observed during the endodormancy phase despite the different dormancy duration. As these two genes play functions related to floral and reproductive development in *A. thaliana*, this could indicate that the early bud break of PL is mainly related to the flowering process to the dormancy release, or even a lighter dormancy that the flowering induction can finish.

Another point is that the differences were observed in the warmer year (2019/20) but not in, the colder year (2018/19) (Figure 3c,d). This could indicate that other possible genes and/or mechanisms could be happening, but they were not detected because the winter was cold enough for the normal bud dormancy cycle. An analysis in a stressful condition (warm temperatures in the critical point of chilling accumulation) could unveil these hidden actors.

The other eight genes selected for quantification assays did not differentiate between the cultivars in the dormancy cycles tested. We suppose they do not directly influence the dormancy duration and progression in the tested conditions (annexes I, II, and III). However, since some of these genes encode members of the AP2/ERF TFs family (e.g., DREB/CBF), it is still possible that they present a more relevant role during a more stressful environment (e.g., low chilling availability, intense heat, or drought, among others).

A preliminary study of DASI associated with dormancy in apple

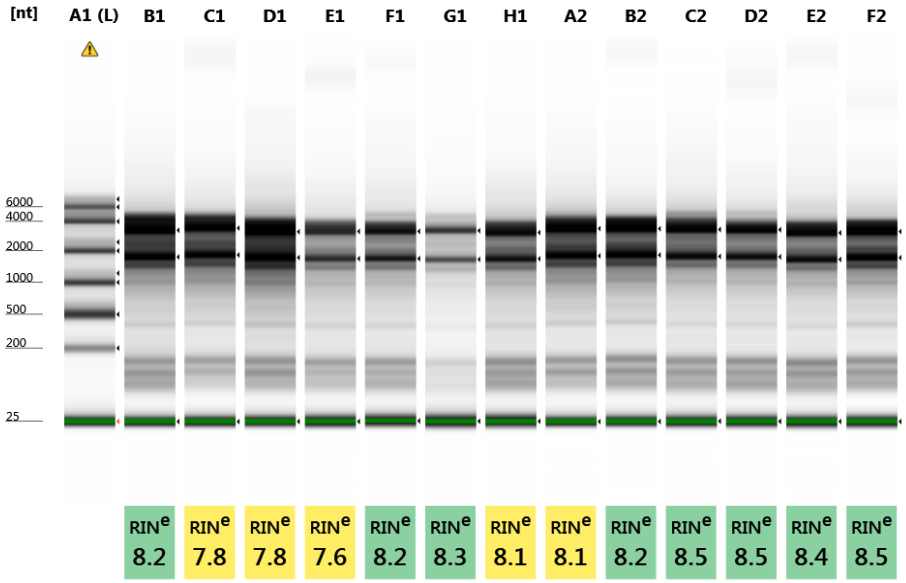
Reproductive phenology is the result of the integration of environmental and endogenous signals into complex genetic networks. Ambient temperature is the main input signal recognized by specialized molecular networks to adapt plant development to seasonal changing environmental conditions [96]. In *A. thaliana*, it has been shown that a number of key floral-regulator genes (mostly encoding MADS TFs) participate in these molecular networks to control flowering time. Remarkably, in *A. thaliana,* the response to ambient temperature is partly modulated by the post-transcriptional regulation of these key genes. In apple, it has been recently proposed that the dormancy cycle is regulated, at least in part, by a GRN formed by MADS TFs. Here, we have hypothesized that the genes encoding these MADS TF and other potential dormancy regulators could be post-transcriptionally regulated by AS. For this reason, a genome-wide analysis to detect DASI during the dormancy cycle of apple was done. In a first attempt, the Tuxedo pipeline [56] was optimized to be used with the apple genome, resulting in the identification of thousands of alternatively spliced gene isoforms. Although the first results were optimistic and implied that Tuxedo's pipeline could be used for the study of AS in apple, we noticed that the prediction of gene isoforms was not correct, and therefore, more effort in optimization of the pipeline is required.

To advance in the study of AS during dormancy in apple, we moved to a direct approach and focused on genes already known to be involved in the apple dormancy cycle. In this regard, *MdMAF2*, *MdFLC-like* and *MdSVPa* seemed to be regulated by AS during dormancy. Remarkably, *MdSVPa* presented potential alternative splicing that could affect its activity. One of the identified isoforms presents an extra small exon (the exon 8) at the side of the 5' UTR region. This potential variation might influence the resulting C-terminal region of the MdSVPa protein and affect its ability to dimerize with other proteins, attach to DNA and/or the recruitment of histone deacetylase complex components [97–99]. Moreover, a transient reduction of the AS containing the exon 8 of *MdSVPa* seems to be associated with the precise moment of transition between endo- to eco-dormancy (Figure 13). This finding enables the possibility that AS of *MdSVPa* plays a role in the control of the dormancy cycle. Whether the AS of *MdSVPa* has an influence on dormancy control, for instance, by affecting the formation of transcriptional complexes previously described [60], requires further investigations.

Final conclusion

In this work, it was possible to observe by transcriptomics analysis that the bud endodormancy release in the apple tree is a process characterized by important deregulation of gene expression. Many of the DEG identified in this study are involved in pathways related to phytohormones, growth, flowering, temperature response and redox signaling, among other cellular identity functions. From this list, we could select a group of candidate genes and quantify them in two cultivars of apple that present distinct CR and HR. In this way, we identified a pair of genes that are related to heat response during endodormancy release and are potentially acting in bud burst induction. Yet, other sets of candidate genes are available for further studies. Moreover, other cultivars could be considered for genetic variability investigation of those candidate genes, serving as a base for future tools for breeding programs to adapt crops to the global climatic changes.

Annex A



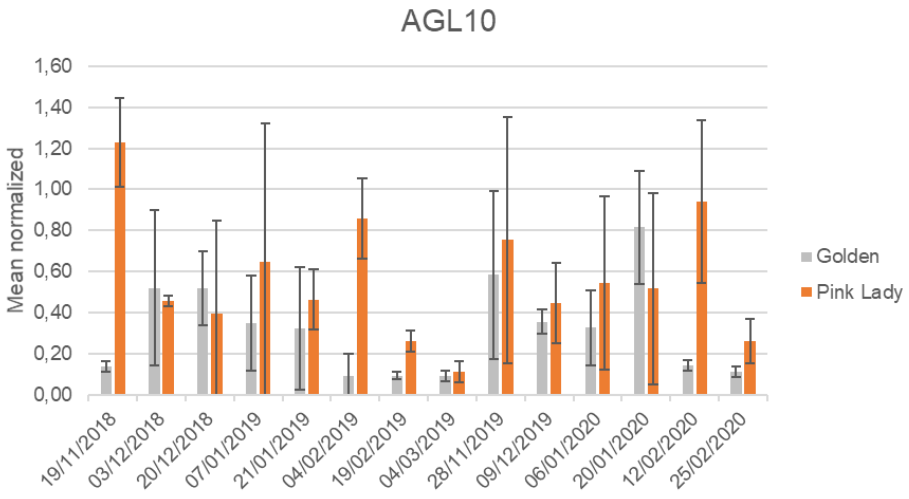
Example of eletropherogram from TapeStation 4200 (Agilent) to evaluate RNA quality. Only samples with RIN over 8.5 were used for sequencing purposes.

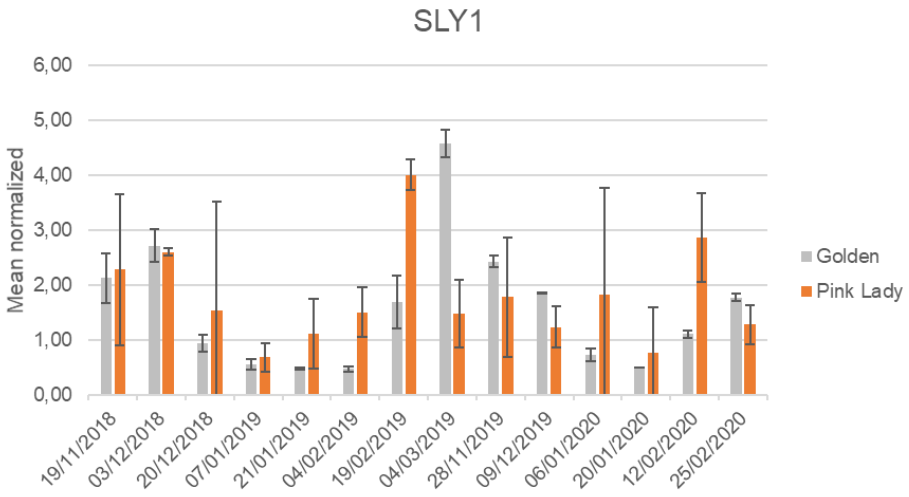
Table 1-1 List of primers and their sequence used in this work

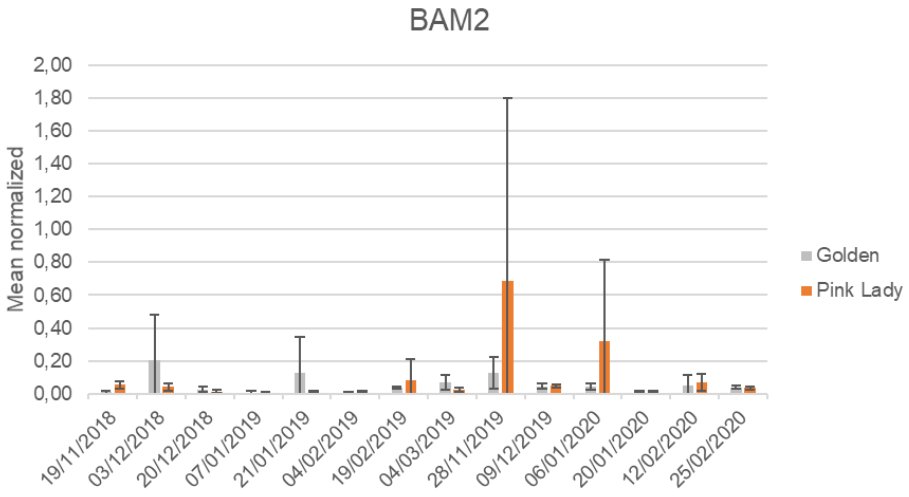
Gene	Code	sequence
MD10G1041100	MdAGL10 foward	GGGGCGAGGGAAGGTAGA
	MdAGL10 reverse	GGACAGCTCACGTGCCTT
MD15G1075800	MdSLY1 Foward	CTGAAAGGGGCGGGGTTT
	MdSLY1 Reverse	GCAGCCGATGTTAGCCCA
MD14G1086100	MdARR9 Foward	CCAGATACGGTGGGATCGC
	MdARR9 Reverse	CGGCCAGTATCAACCAGCA
MD08G1241900	MdBAM2 Foward	AATTCCGGGCGAGTTGGG
	MdBAM2 Reverse	GCCCATCCAATTCGCAGC
MD00G1036300	MdHB7 Foward	CACACTCGCACACTCCCA
	MdHB7 Reverse	CCTTGTAGTGGCAGCTCCC
MD04G1067900	MdCBF2 Foward	CAGTGGATGTCAGGCGGG
	MdCBF2 Reverse	GCTCCGCACCTCCACAAT
MD08G1035400	MdBZR1 Foward	CCCAGTCCCACCCGTTTC
	MdBZR1 Reverse	ATTGGGTTGGGGCGTCTG
MD09G1264800	MdRGA-like 2 Foward	GGGTCTAATTCCGGCGGG
	MdRGA-like 2 Reverse	GGACGAGTTGGACACCGG
MD15G1064600	MdANT Foward	CCGAGGCGTGACAAGACA
	MdANT Reverse	TGCGCCACGGAACTTGAT
MD14G1241400	MdHB22 Foward	AGAGGATGAGCAGCGGGA
	MdHB22 Reverse	GCCGGTGGATGCAGAAGT
MD08G1215900	MdWD40 Foward	GGATTTACTGTGTTGGTGAAG
	MdWD40 Reverse	TGCCAATTACCTCCTTTTCGTG
MD16G1219000	MdMDH Foward	CGTGATTGGGTACTTGGAAC
	MdMDH Reverse	TGGCAAGTGACTGGGAATGA

Annex I

RT-qPCR profiles of AGL10, SLY1 and BAM2 on Golden delicious and Pink lady at 2018-19 and 2019-20 winter cycles

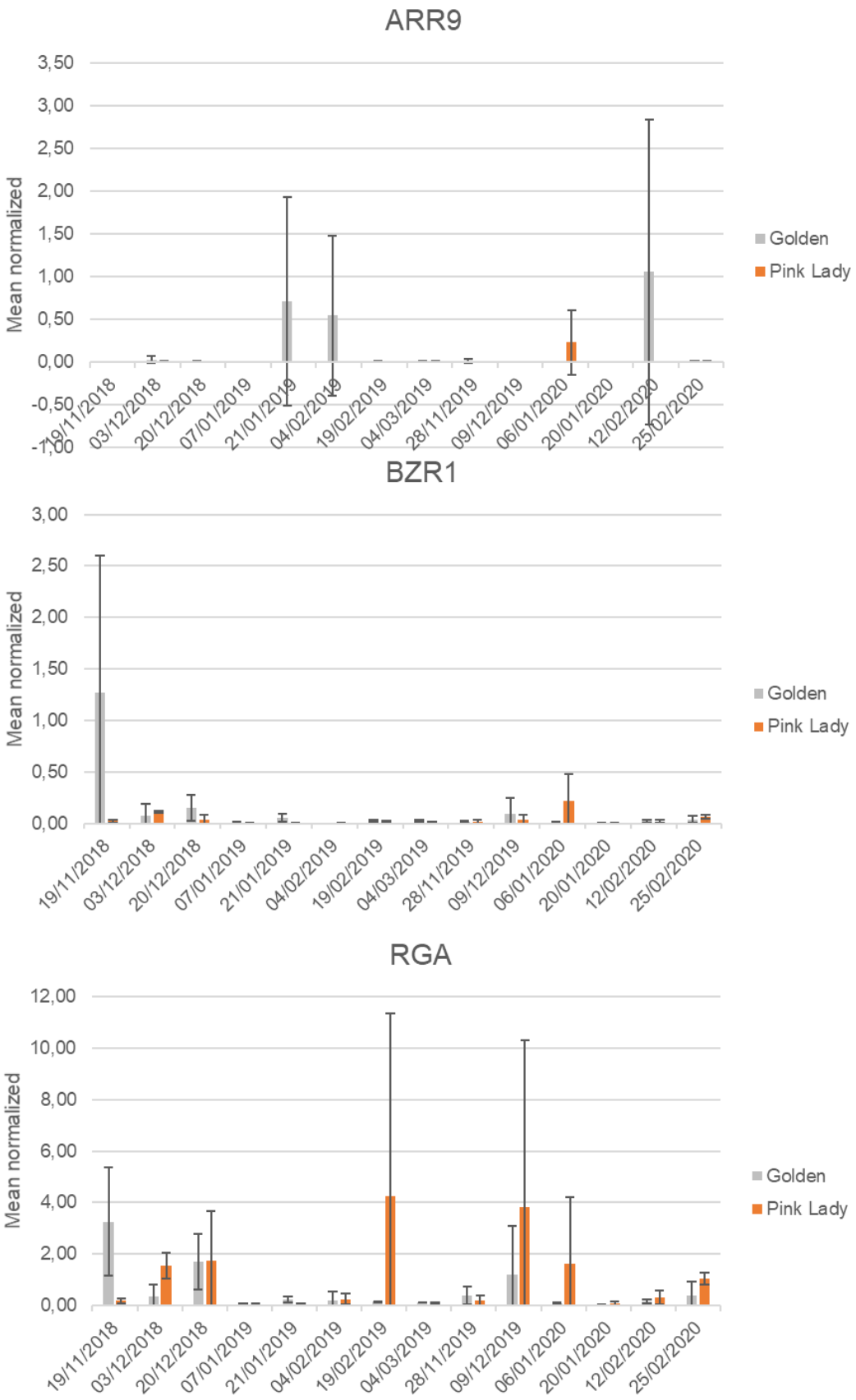






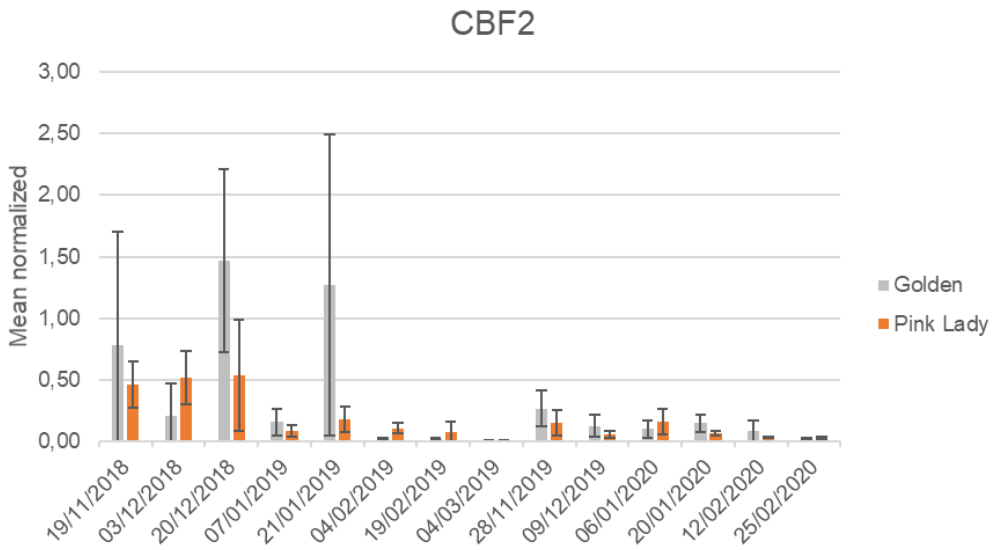
Annex II

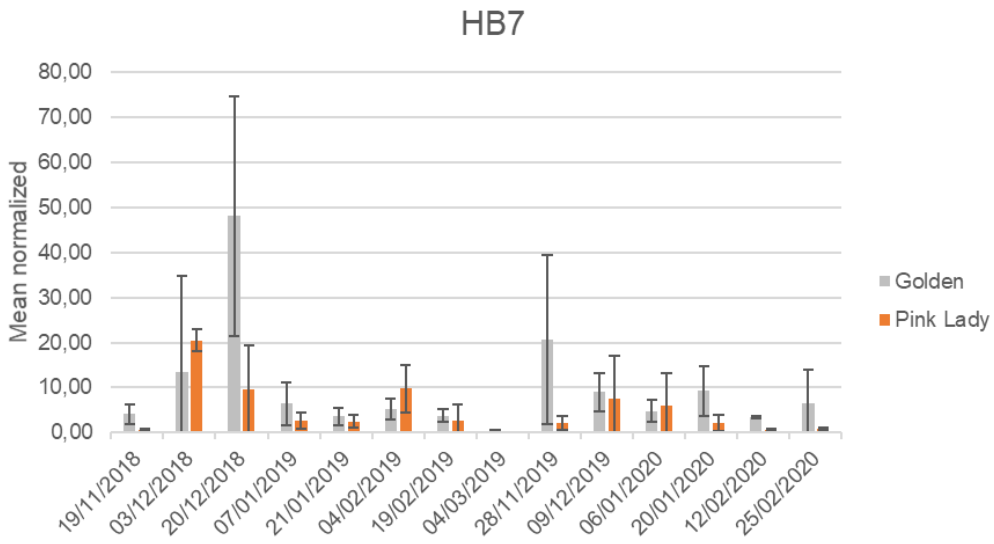
RT-qPCR profiles of ARR9, BZR1 and RGA on Golden delicious and Pink lady at 2018-19 and 2019-20 winter cycles



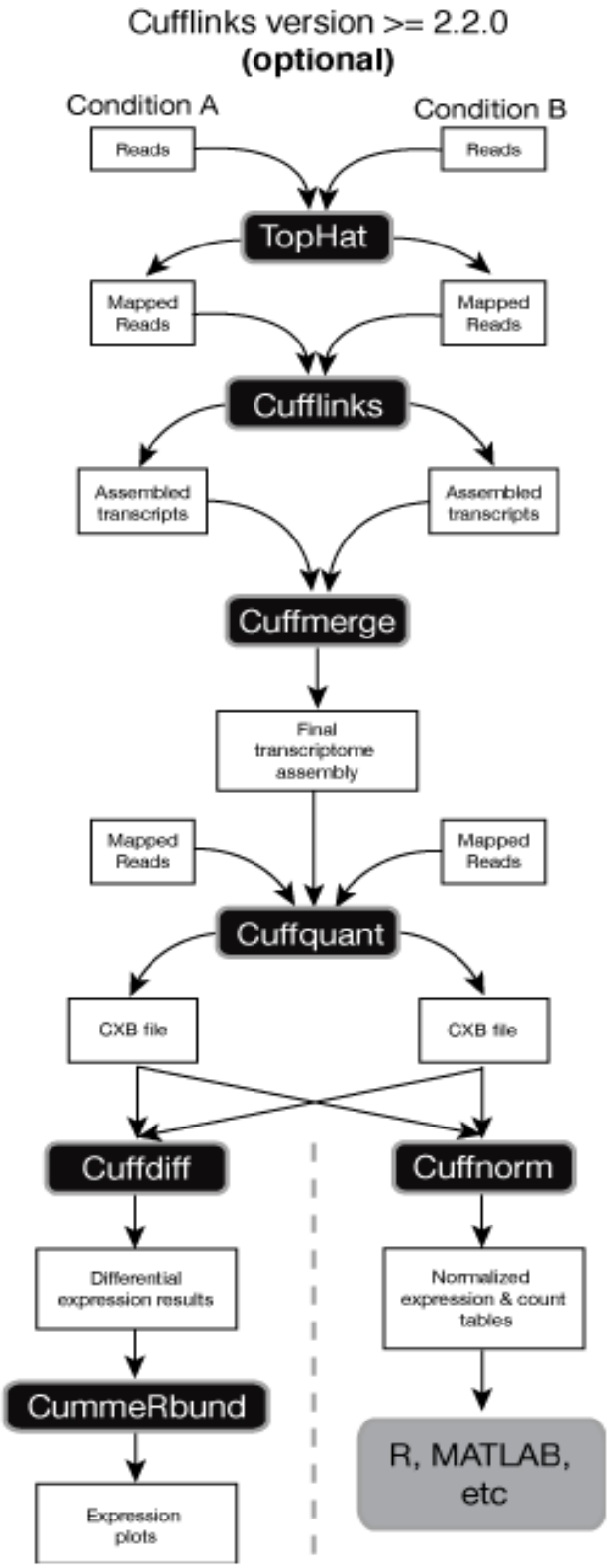
Annex III

RT-qPCR profiles of CBF2 and HB7 on Golden delicious and Pink lady at 2018-19 and 2019-20 winter cycles





Annex IV - Pipeline used for alternative splicing study on raw data from sequencing



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CHAPTER 2

The effect of Temperature and Oxidative Stress during Bud Dormancy

ABSTRACT

Bud dormancy progression and release are highly affected by environmental temperatures. Warm temperatures during endodormancy (when chilling is required) or chilling in ecodormancy (when heat is required) are known for disturbing bud phenology and, consequently, flowering and fructification. However, how temperature sensing and signaling pathways are genetically regulated to modulate the dormancy cycle is poorly understood. Remarkably, it has been proposed that Reactive Oxygen Species (ROS) may act as signaling molecules for dormancy and their abundance is affected by changes in temperature. Indeed, the commonly used bud breaker cyanamide hydrogen causes redox stress that leads dormant trees to bud break even before the fulfillment of their CR. Furthermore, crosstalk between temperature and ROS signaling pathways have been proposed in the regulation of seed and bud dormancy and the vernalization flowering pathway in *A. thaliana*.

In Chapter 1, a gene ontology analysis of differentially expressed genes during the dormancy cycle suggested the involvement of temperature and oxidative stress signaling pathways in endodormancy release. To further characterize the role of ROS and temperature in dormancy in Chapter 2, I have selected 26 genes that were differentially expressed in the RNA-seq experiment to test their behavior under different temperature treatments (4 °C and 25 °C) and to ROS (H_2O_2). ROS treatments increased bud break percentage in mid-endodormancy and just after endodormancy release. ROS-induced bud break at mid-endodormancy was accompanied by the down-regulation and up-regulation of the apple homologs of the *A. thaliana FLOWERING LOCUS C* (*MdFLC-like*) and *CRINKLY 4* (*MdCR4*) genes, respectively. After endodormancy release, the ROS treatment led to the down-regulation and up-regulation of the genes annotated as *Gibberellin Responsive Protein 6* (*GRP6*) and *Iron-Sulfur Cluster Scaffold Homolog* (*NFU1*).

In regard to the temperature-response assay, I have observed significant changes in gene expression at both high and low temperatures. Many of the studied genes were responsive to temperature changes in all dormancy phases, while others only responded to heat at specific phases. Notably, *FLC*-like was down-regulated and up-regulated in response to warm and cold temperatures, respectively. I also found that warm temperatures induced the expression of the apple homolog of *DICER-Like*

3 (*DCL3*), which in *A. thaliana* is required for endogenous RDR2-dependent siRNA formation.

The results of this Chapter suggest a combination of redox signaling, acclimation and developmental responses to temperature could be part of the genetic (and epigenetic) regulation of dormancy, in addition to the already demonstrated hormonal and transcriptional regulations. We thus open new perspectives to understand bud dormancy and prepare new plant material better adapted to the near future under climatic changes.

RÉSUMÉ

La progression et la levée de la dormance des bourgeons sont fortement affectées par les températures environnementales. Les températures chaudes pendant l'endodormance (quand le froid est requis) ou le froid en écodormance (quand la chaleur est requise) sont connues pour perturber la phénologie des bourgeons et, par conséquent, la floraison. Cependant, la façon dont les voies de détection et de signalisation de la température sont régulées génétiquement pour moduler le cycle de dormance est mal comprise. Il a été proposé que les espèces réactives de l'oxygène (ROS, Reactive Oxygen Species) puissent agir comme des molécules de signalisation pour la dormance et que leur abondance soit affectée par les changements de température. En effet, l'hydrogène cyanamide qui est couramment utilisé en vergers, provoque un stress redox qui conduit les arbres dormants au débourrement avant même l'accomplissement de leur CR. De plus, une interaction entre les voies de signalisation de la température et des ROS a été proposée dans la régulation de la dormance des graines et des bourgeons, et dans la voie de floraison de vernalisation chez *A. thaliana*.

Dans le chapitre 1, une analyse de l'ontologie des gènes exprimés de manière différentielle pendant le cycle de dormance a suggéré l'implication des voies de signalisation de la température et du stress oxydatif dans la levée de l'endodormance. Pour caractériser davantage le rôle des ROS et de la température au cours de la dormance, j'ai sélectionné 26 gènes qui étaient exprimés de manière différentielle dans les données de séquençage d'ARN pour tester leur comportement sous différents traitements thermiques (4 °C et 25 °C) et ROS (H_2O_2). Les résultats de ces traitements sont présentés dans ce chapitre 2. Les traitements aux ROS ont augmenté le pourcentage de débourrement au milieu de l'endodormance et juste après la levée de l'endodormance. Le débourrement induit par les ROS à mi-endodormance s'accompagnait d'une régulation à la baisse et d'une régulation à la hausse des homologues de pomme des gènes *A. thaliana FLORING LOCUS C* (*MdFLC-like*) et *CRINKLY 4* (*MdCR4*), respectivement. Après la levée de l'endodormance, le traitement ROS a conduit à la régulation négative et à la régulation positive des gènes annotés comme *Gibberellin Responsive Protein 6* (*GRP6*) et *Iron-Sulfur Cluster Scaffold Homolog* (*NFU1*).

En ce qui concerne le test de réponse à la température, j'ai observé des changements significatifs dans l'expression des gènes aussi bien à des températures élevées que basses. De nombreux gènes étudiés ont réagi aux changements de température dans toutes les phases de dormance, tandis que d'autres ne répondaient à la chaleur qu'à des phases spécifiques. Notamment, le gène type-*FLC* a été régulé à la baisse et à la hausse en réponse aux températures chaudes et froides, respectivement. J'ai également découvert que les températures chaudes induisent l'expression de l'homologue de *DICER-Like 3* (*DCL3*) du pommier, qui, chez *A. thaliana*, est nécessaire à la formation endogène d'ARNsi dépendant de RDR2.

Les résultats de ce chapitre suggèrent que la combinaison de la signalisation redox, de l'acclimatation et des réponses développementales à la température pourrait faire partie de la régulation génétique (et épigénétique) de la dormance, en plus des régulations hormonales et transcriptionnelles déjà démontrées. Nous ouvrons ainsi de nouvelles perspectives pour comprendre la dormance des bourgeons et préparer un nouveau matériel végétal mieux adapté face aux changements climatiques, dans un futur proche.

1 Introduction

Dormancy is an essential adaptive trait for temperate fruit crops. In the apple tree, it corresponds to cold acclimation processes induced by low temperatures [1]. The autumn low-temperature signals induce bud-set and endodormancy establishment. Throughout continuous chilling accumulation, endodormancy is released and ecodormancy is achieved (see the general introduction and chapter 1 for definitions). The amount of chilling to be accumulated to reach endodormancy release and ecodormancy is genotype/variety dependent. Similarly, at the ecodormancy stage, growth is only restored after an appropriate period of exposure to warm temperatures in a genotype-dependent way [2]. In this context, the bud dormancy is dependent on the availability of cold or warm temperatures at the right time, meaning that cold temperatures are required to release endodormancy, but heat waves during this period are not beneficial for dormancy progression. Additionally, the warm temperatures are essential at the ecodormancy stage to properly induce bud break and flowering and the occurrence of cold waves during that period can block the flower development or even the flowering process, thus leading to the loss of flowers and poor production. The dependency of dormancy progression and flower development on temperatures and its importance for fruit crops, especially in the Rosaceae family, has motivated studies on dormancy and its relation to temperature fluctuations.

Moreover, the Chilling Requirement (CR), measured in Chilling Hours (CH), is required to overcome endodormancy, and the Heat Requirement (HR) is required to overcome the ecodormancy, being both genetically defined. The studies of those requirements and the related genetic regulations have started with the discovery of a peach evergreen mutant in which no bud dormancy was observed. Genetic studies have shown that this mutation was caused by a deletion in the region of the genome that contains several genes encoding MADS-box transcription factors (TF) that therefore were named *DORMANCY-ASSOCIATED MADS BOX* genes (*DAM* genes). It has been recently proposed that DAM TFs act in concert with other MADS TFs in a gene regulatory network (GRN) that controls dormancy in fruit trees [3]. This GRN is, in turn, modulated by changes in temperature through the transcriptional regulation of its components [4–6]. Low temperatures not only modulate gene expression within dormancy-related GRNs but also alter the production and distribution of

phytohormones. Indeed, regulatory feedback between dormancy-related MADS TFs and hormones is believed to participate in abiotic stress signaling to shape tree acclimatization and adaptation in response to low temperatures [4].

During winter, trees must cope with a variety of abiotic stresses, including extreme temperatures and drought stresses. They respond to these stresses through the accumulation of signaling and protective substances [7][7]. Notably, changes in ions fluxes, callose accumulation, phytohormones, and reactive oxygen species (ROS) are the first responses induced to tackle the stresses through signaling cascades. These signaling cascades are governed by specific TFs that interact with the cis-acting elements included in the promoter regions of stress-responsive genes [8]. For example, APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) represents a family of TFs involved in the regulation of various stress responses, in which they also respond to hormones [9]. Part of this large TF family includes cold-related genes such as the *COLD-INDUCIBLE C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTORS* (*CBF/DREB*) [10]. Apart from their participation in specific stresses, AP2/ERF TFs are also involved in a wide range of stress tolerance processes, enabling them to form an interconnect into regulatory networks. Additionally, many AP2/ERF TFs respond to the plant hormones such as abscisic acid (ABA) [11–13] and ethylene (ET) [14,15] and are involved in the activation of ABA and ET dependent and independent stress-responsive genes. Moreover, some AP2/ERFs are implicated in the growth and developmental processes mediated by gibberellins (GAs), cytokinins (CK), and brassinosteroids (BRs) [16,17]. Also related to cold and desiccation, there is a group of TFs known as No Apical Meristem (NAC) and Salt tolerance zinc finger (STZ). In *A. thaliana*, ZAT10 (A. thaliana STZ) is hardly expressed under non-stress conditions and is controlled by the relation of ROS and ascorbate peroxidase (APX) activity [18].

Strikingly, oxidative stress and hormone signaling participate in the release of dormancy in seeds [19]. Similarly, the literature suggests that redox and hormone signaling participate in bud dormancy release in trees [20]. Indeed, the production of H_2O_2 and other ROS molecules may act as signals for dormancy release in buds. Supporting this idea, the application of exogenous H_2O_2 can substitute for chilling to activate the breaking of bud dormancy [19]. Also, the hormonal balance between ABA and GA may mediate the control bud break, acting as a growth repressor and activator,

respectively [4]. Interestingly, ROS is involved in the regulation of hormonal pathways by directly acting on hormone redox reactions and/or by affecting the activity of hormone-signaling components [21]. However, the role of TFs in the genetic regulation of temperature and stress perception, as well as their integration in hormonal and ROS signaling networks during bud dormancy, is unclear in fruit tree species such as the apple tree.

In this Chapter, I aim to study the potential role of TFs in the temperature- and redox-mediated control of dormancy. A list of candidate TFs was obtained from the GO enrichment presented in Chapter 1. This list contains genes encoding TFs (or DNA binding proteins) that are differentially expressed during the dormancy cycle (Chapter 1, Figure 5b) with predicted GO function related to drought and cold tolerance, flowering time control, hormonal signaling, response to warm temperatures and redox homeostasis.

2 Material and Methods

Phenology

Branches 30-40 cm long, presenting approximately ten shoots with terminal floral buds and aged two years at least, were collected and used for Tabuenca [15] and bud break tests. Those branches were selected on the same group of trees from the ‘Golden delicious’ cultivar from RNA-Seq (more detail in Chapter 1) every two weeks during the dormancy cycle of 2020/2021. For the Tabuenca test, three shoot segment replicates, each composed of five buds were tested. The bud break test used four replicates of ten cuttings, each composed of a single bud and set in water pots in forcing conditions for 30 days at 25 °C with a photoperiod of 16h/8h light/dar.

Testing temperature effect on bud break

Branches similar to those selected for the forcing tests (30-40 cm, presenting approximately ten terminal floral buds, aged two years at least) were selected on trees located in the same rows of the experimental orchard. The number and length of the shoots were also similar in this experiment to the previous one. The shoots were put in two contrasting temperatures: 25 °C or 4°C (photoperiod of 16h/8h light/dark) for 24h and then nitrogen frozen. Three pools of three buds were considered for each

sampling. This test was done every third week during the 2020/21 dormancy. The samples harvested were used for gene expression assays.

Testing redox status effect on Bud break

Testing the concentration effect of H_2O_2

From January 2020, when the endodormancy period was achieved, branches presenting the same characteristics as for the forcing tests were collected (30-40 cm, presenting approximately ten terminal floral buds aged two years at least) from trees located in the same rows. Shoots (which length, how many buds) were treated with hydrogen peroxide (H_2O_2) at five different concentrations (1, 5, 10, 20 and 40 mM) and compared to a control group (0 mM H_2O_2). Each group had at least 30 buds. The buds were submerged in water containing H_2O_2at the concentration corresponding to each treatment for 1h. Shoots were then removed from the water but kept yet moist for another 1h, with their bottoms always maintained in water. Then the branches were washed with water only and placed in a 4°C chamber for 24h. After this period, three pools of three buds per treatment were sampled, and the other buds were transferred to the forcing conditions to determine their dormancy state.

H_2O_2 on dormancy cycle test

Branches from the 2020/2021 dormancy cycle (30-40 cm, presenting approximately ten terminal floral buds aged two years at least) from the same row of phenological determination were treated with the H_2O_2 concentration considered in the previous test as optimal for the expected effect. A new set of branches was treated with 10 mM of H_2O_2, while another set was considered as a control group (0 mM H_2O_2). The treatment was applied every three weeks from 12/11/2020 (early endodormancy) to 23/02/2021 (late ecodormancy). Each group had at least 30 buds. The buds were treated as previously: submerged in water with H_2O_2 for 1h, removed from the water but kept yet moist for another 1h (bottoms of branches always in water). Then the branches were washed with water and then placed in a 4°C chamber for 24h. After this period, three pools of three buds per treatment were sampled, and the other buds were transferred to the forcing conditions for phenology measurement.

Expression Analysis by RT-qPCR

RNA extraction

All samples previously selected based on their phenological state had their total RNA extracted from ~100 mg bud powder. A 'Spectrum Plant Total RNA Kit' from Sigma was used for total RNA sequencing. The quality of the RNA was verified in a TapeStation 4200 (Agilent Genomics).

cDNA production

500ng RNA of samples (in 8µL water) were treated with 'RQ1 RNase-Free DNase' (Promega), and then the reverse transcriptions were done with 'SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase' (ThermoFisher). Both procedures were performed according to the manufacturer's instructions.

qRT-PCR

Quantitative PCRs were performed using the kit' LightCycler® 480 SYBR Green I Master' in the 'LightCycler 480 - Multiwell Plate 384' (Roche Diagnostics GmbH) according to the manufacturer's instructions. Primers were designed in the program Primer3 [16] (see table of primers in Supplementary data). The genes *MdMDH* and *MdWD40* were used as a reference for relative gene expression calculus. A group of selected genes (see table 1) and the so-called dormancy marker genes (Table 2) were studied. The relative expression levels were calculated by the $2^{-\Delta Ct}$ [22]

Statistical analyses

For the phenological evaluation from the 'H_2O_2 on dormancy cycle test', the chi-squared test was used. For the RT-qPCR, the treated was compared to the control in paired t-test.

3 Results

Selection of genes potentially involved in redox- and temperature-mediated control of dormancy in apple

The RNA-seq experiment performed in Chapter 1 resulted in the identification of several DEG between endodormancy and ecodormancy phases. The list of DEG was screened for genes with annotated functions (mainly) related to response to temperature and ROS as well as flowering, dormancy and hormone signaling. Most of these genes were also chosen for their molecular function related to transcriptional regulation (e.g., TF). The list of the selected genes is presented in Table 1. In the next sections, the expression of the 26 selected genes and ten dormancy-marker genes (Table 2) in response to oxidative stress and contrasted temperatures was studied by RT-qPCR.

Table 2-1 List of candidate genes selected from results of Chapter 1

GDDH13	TAIR	Description	GO	code
MD00G1036300	AT2G46680	homeobox 7	Cold (ABA/drought)	HB7
MD00G1076100	AT5G07330	unknown protein	heat and ROS	NFU1
MD02G1195700	AT2G45690	shrunken seed protein (SSE1)	ROS	SSE1
MD02G1307700	AT3G08030	Protein of unknown function, DUF642	Dormancy	HA2
MD03G1057400	AT2G38470	WRKY DNA-binding protein 33	Cold (salt stress and ROS)	WRKY33
MD03G1222700	AT4G27410	NAC (No Apical Meristem) transcriptional regulator	Cold (drought and ABA)	NAC
MD03G1273800	AT1G54690	gamma histone variant H2AX	flowering repression	H2AX
MD04G1040500	AT3G59420	crinkly4	flowering	CRINKLY
MD04G1067900	AT4G25480	dehydration response element B1A	Cold/ABA	CBF2
MD05G1204300	AT5G44210	erf domain protein 9	transcription regulation	ERF9
MD08G1035400	AT1G75080	Brassinosteroid signalling positive regulator	flowering and ABA	BZR1
MD08G1086500	AT1G27730	salt tolerance zinc finger	Cold (ROS tolerance)	STZ
MD08G1241900	AT3G49670	Leucine-rich receptor-like protein kinase family protein	Dormancy	BAM2
MD09G1264800	AT3G03450	RGA-like 2	Dormancy/gibberellins	RGA-like2
MD10G1041100	AT2G03710	K-box region and MADS-box transcription factor	Cold (flowering/MADS-box)	AGL10
MD12G1227200	AT5G27320	alpha/beta-Hydrolases superfamily protein	Dormancy	GID1C
MD14G1086100	AT3G57040	response regulator 9	vernalization	ARR9
MD14G1165600	AT3G48430	relative of early flowering 6	flowering	REF6
MD14G1200300	AT1G74670	Gibberellin-regulated family protein	Dormancy/gibberellins	GPR6
MD14G1241400	AT4G24660	homeobox protein 22	Dormancy	HB22
MD15G1047500	AT3G43920	dicer-like 3	flowering	DICER3
MD15G1064600	AT4G37750	Integrase-type DNA-binding superfamily protein	flowering	ANT
MD15G1075800	AT4G24210	F-box family protein	Dormancy/gibberellins	SLY1
MD15G1209400	AT2G26150	heat shock transcription factor A2	Cold (ROS)	HSFA2
MD16G1058900	AT1G69180	transcription factor YABBY family protein	flowering	CRC
MD17G1132100	AT3G59030	MATE efflux family protein	Dormancy	TT12

Table 2-2 List of Dormancy-marker genes

code	GDDH13	GO
MdCBF4	MD04G1067800	cold (ABA/drought)
MdDAM1	MD15G1384500	Cold (flowering/MADS-box)
MdDAM4	MD08G1197300	Cold (flowering/MADS-box)
MdDAMb	MD15G1384600	Cold (flowering/MADS-box)
MdEBB1	MD13G1101100	Cold (flowering/MADS-box)
MdFLC-like	MD09G1009100	Cold (flowering/MADS-box)
MdMAF2	MD05G1037100	flowering
MdMDAM2	MD08G1196900	Cold (flowering/MADS-box)
MdSVPa	MD01G1038600	Cold (flowering/MADS-box)
MdSVPb	MD15G1313200	Cold (flowering/MADS-box)

3.1 Study of expression changes related to oxidative stress

The GO enrichment analysis made on the RNA-seq data highlighted a possible role of genes related to oxidative stress during the apple dormancy cycle. Redox signaling, together with hormones, might operate in the transition from endodormancy to ecodormancy in fruit trees [19]. To test this hypothesis, a redox test was planned in which apple tree branches with endodormant buds would be treated with H_2O_2 (a major redox metabolite [23]), and the gene expression and phenology would be analyzed. As the redox state is sensitive machinery to stress sensing and response, an initial test was performed to choose the most effective H_2O_2 concentration with respect to the effect on dormancy release. Among the six concentrations tested (control 0, 1, 5, 10, 20 and 40 mM of H_2O_2; Figure 1), the 10 mM H_2O_2 presented a statistically significant higher bud break than the control (0 mM H_2O_2) according to paired t-test. The fact that higher concentrations did not provoke bud break suggests possible toxicity (20 and 40 mM H_2O_2). The concentrations of 1 and 5 mM H_2O_2 presented no significant or small percentage of bud break compared to the control.

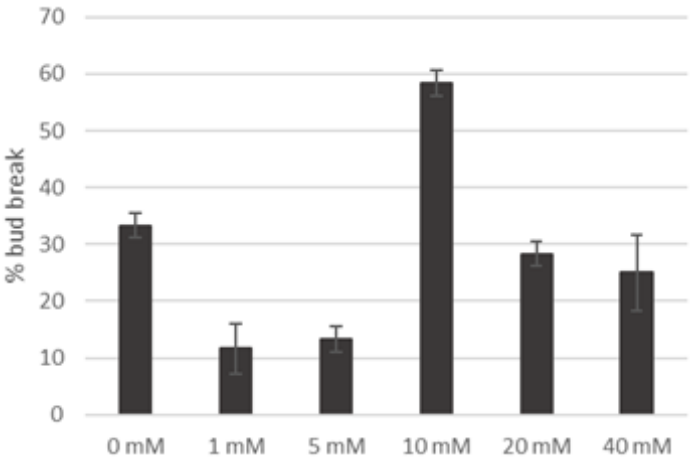


Figure 2-1 Bud break percentages 30 days after H2O2 treatments with diverse concentrations: 0 mM H2O2 (control), 1, 5, 10, 20, and 40 mM H2O2

Aiming to identify the effect of oxidative stress in gene expression during endodormancy, the concentration of 10 mM H_2O_2 was chosen for its effect on bud break rate. The next test focused on the effect of this H_2O_2 concentration on dormancy release and bud break, i.e., effects on phenology and the expression of genes related to dormancy. We thus examined the bud break progression of buds treated with 10 mM of H_2O_2 in comparison to control (no H_2O_2) during the dormancy cycle (from mid-endodormancy until ecodormancy). A statistically significant difference in the bud break percentage was found between treated and control conditions at the second and at the fourth time point. Once each one of the time -points is in a diverse dormancy state (endodormancy for the second point and ecodormancy for the fourth point), there is no relation between the dormancy state and redox stress effect over phenology or the pathways that lead to phenological different are different in each state (Figure 2a).

The level of expression of the dormancy marker genes and the selected genes were measured in five time-points covering the phases of endodormancy (dates: 12/11/2020, 18/12/2020 and 11/01/2021) and ecodormancy (dates: 08/02/2021, 23/02/2021). Significant differences were observed at the second time point for two genes, *FLC*-like lower and *CRINKLY,* which expression was higher in the treated group than in the control one. At the fourth time point (with differential phenology in ecodormancy), significant differences were found in the expression of *NFU1* and *GRP6,* which expression was higher and (lower respectively in the treated group compared with the control. In the last time-point (ecodormancy, no significant

phenological difference), the gene *SSE1* presented significantly lower expression in the treated group than in the control. A scheme summarizing those results is presented in Figure 2b. More detailed expression profiles can be found in Annex I.

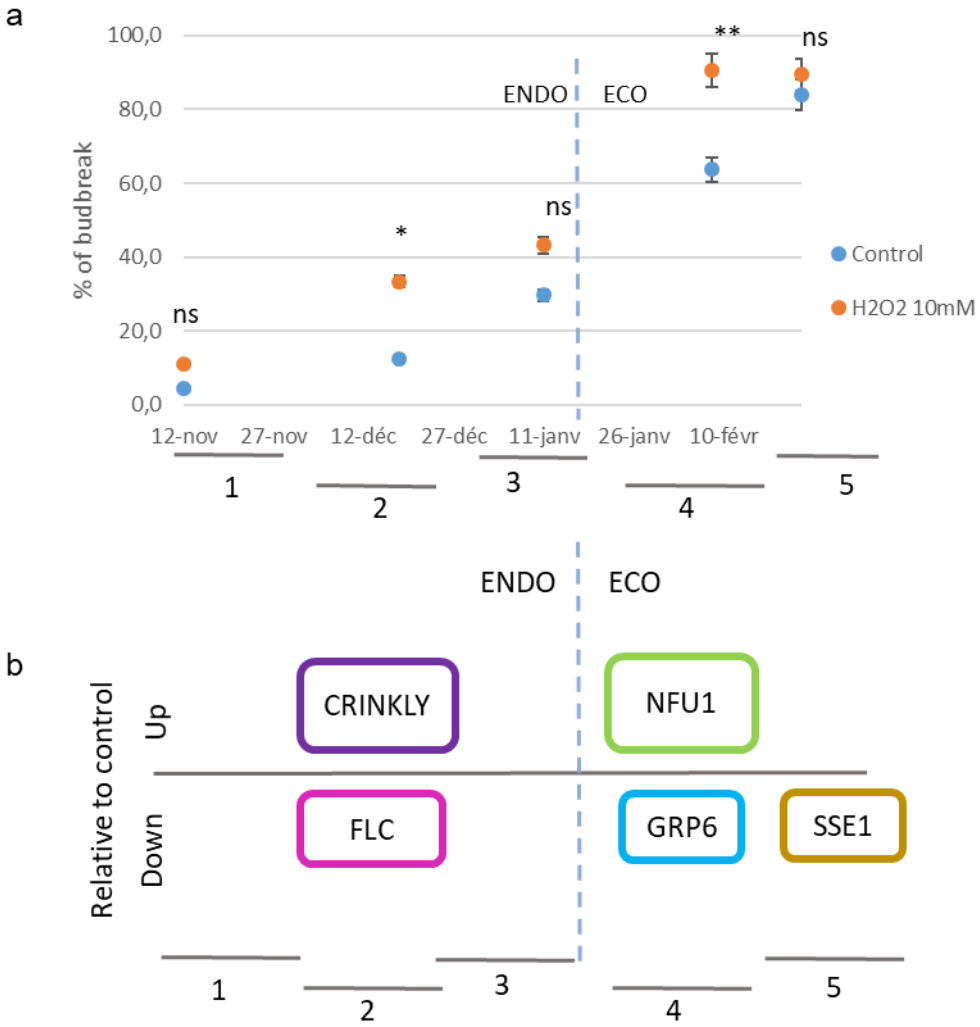


*Figure 2-2 a) Comparison of bud break percentages in treated (in orange) and control (in blue) samples at consecutive time points during the dormancy cycle. * $p \leq 0.05$ ** $p \leq 0.01$ represents significant difference between conditions according to a Khi-two test. b) Simplified representation of relative expression of the tested genes (markers and candidates) in comparison to control. Complete profiles of expression are available at Annex I.*

3.2 Study of expression changes related to contrasted temperatures

Dormancy marker genes

The expression of genes belonging to the group considered dormancy-markers (table 2) was tested under contrasted temperature conditions (i.e., 25 °C vs. 4 °C). Some dormancy-marker genes presented a differential expression between the temperature treated (25 °C or 4 °C) and the field control samples (Figure 3). *SVPa* is upregulated in the samples treated at 25 °C, at the third and fourth time-points, i.e., during the endo-ecodormancy transition. For samples treated at 4 °C, *SVPa* is also upregulated from the first time point up to the fourth time point, the first ecodormancy point. *CBF4* presents down-regulation at 25 °C at all time-points and up-regulation from second until last time point at 4 °C. The other three genes, *DAM2*, *DAM4* and *DAMb*, present differential expression against the reference from the field. *DAM2* and *DAM4* were down-regulated at 25 °C only at the third time point, i.e., before the endodormancy release, while *DAMb* was down-regulated from the third time-point (the same last endodormancy time point as *DAM2* and *DAM4*) until the fifth time point, the last point in ecodormancy. *FLC*-like was up-regulated in the samples treated at 4 °C in the third time point (endodormancy release) and down-regulated in samples treated at 25 °C in the fourth time point (start of ecodormancy). *MAF2* and *EBB1* present upregulated at the third and fourth points at 4 °C (the last endodormancy point and the first ecodormancy point). The dormancy-marker genes *DAM1* and *SVPb* did not present differential expression in any of the samples and whatever the temperature treatments (Figure 3).

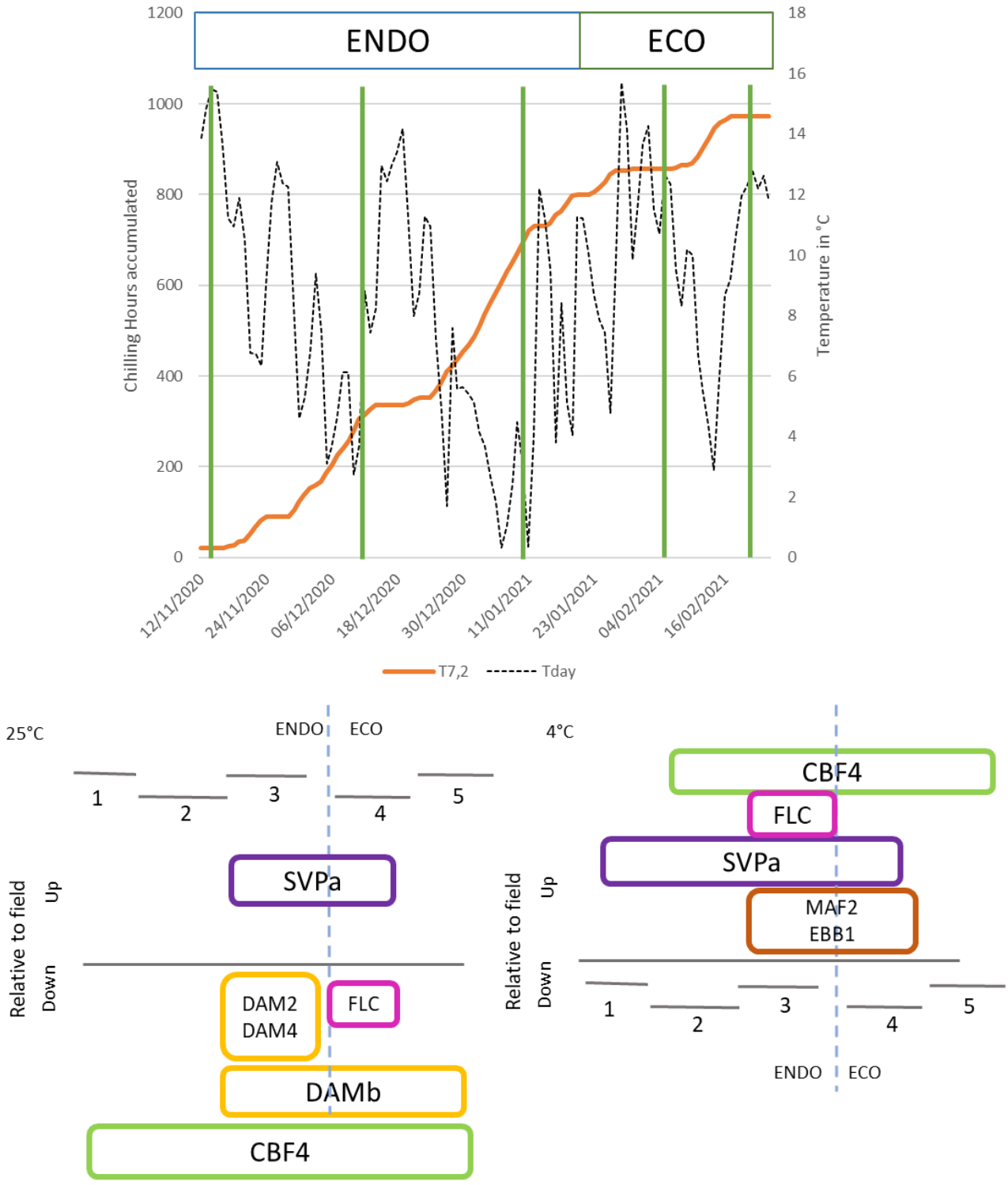


Figure 2-3

Upper part: Temperature evolution during the bud dormancy cycle 2020/2021. Orange line represents the chilling hours accumulated (<7,2°C), the black dotted line shows the daily average temperature (in °C). The vertical green lines represent the dates of sample harvesting for temperature treatment test. Phenological stages determined by Tabuenca test is displayed on top of the Figure (ENDO or ECO).

Bottom part: Simplified representation of expression profiles of markers-genes after temperature test (25°C or 4°C) in comparison with same candidate-gene expression in samples from field. Numbered horizontal bars represent the time-points: three in endo- and two in eco-dormancy.

Candidate genes

Some of the candidate genes that were identified by bioinformatics were expected to be part of the molecular networks regulating the dormancy cycle mediated by temperature. Studying their response to changes in temperature is expected to shed light on their role in the dormancy cycle control. The expression of the selected candidate genes (Table 1) was measured by qPCR in both treated conditions and also in field samples that were used as external controls. The genes classified as qualitatively up- or down-regulated in relation to reference (field samples) are listed in Annex 1. A summary of the expression analyses is shown in Figure 4. Detailed expression data can be found in Annex II. Among those genes, some react to temperature independently of the phase of dormancy, such as *CBF2v2*, *DICER3*, *REF6*, *STZ*, *HB7* and *CRINKLY*. Other genes react to both temperatures, such as *REF6,* which is upregulated in both treatments or show an opposite reaction, such as *CBF2v2,* which is down-regulated at 25 °C and up-regulated at 4 °C. Other groups of genes showed changes in expression in response to temperature treatments only at particular dates during dormancy. Genes from one of these groups were differentially expressed from the second time point (endodormancy yet) until the last time point (ecodormancy). In this group, we found *ARR9*, *HB22*, *BAM2*, *SSE1*, *NFU1*, *AGL10 MADS TF* and *SYL1* that are upregulated in the samples treated at 25 °C. For 4 °C treated samples, *NAC*, *NFU1*, *HSFA2*, *AGL10,* and *ERF9* were found upregulated in the same time points (from the second until the last one). Some genes did not show a temperature-specific response and showed changes in expression independently of the used temperature (e.g., *NFU1* and *AGL10*). Another group of genes started to be upregulated from the third time point (end of endodormancy) until the last time point in ecodormancy. In this group, we found *RGA* and *CRC* in samples treated at 25 °C and *HA2*, *SSE1*, *CRC* and *SYL1* in samples treated at 4 °C. In this group, *CRC* responded to any temperature during the indicated period. Focusing on genes that present upregulation specifically around the transition from endodormancy to ecodormancy, we found the genes *HA2*, *HSFA2*, *ANT*, *CRINKLY* and *HB7* for samples treated at 25°C. On 4°C treated samples, *ANT* and *TT12*. *BZR1* and *NAC* also presented differential expression at a specific time point, but no direct connection to endodormancy release was found.

4 Discussion

4.1 The redox homeostasis potentially acts in direct interaction with cold pathways in endodormancy

ROS homeostasis is a major actor in the plant stress signaling mechanisms, especially in seed dormancy, and it potentially contributes to the tree bud dormancy regulation [24]. In perennial crops, different pathways involving ROS signaling may operate during bud dormancy [19]. Some of those pathways can be activated to induce bud burst, especially by chemicals such as hydrogen cyanamide, which has been commonly used for synchronizing bud burst and for promoting crop stability production [25–27].

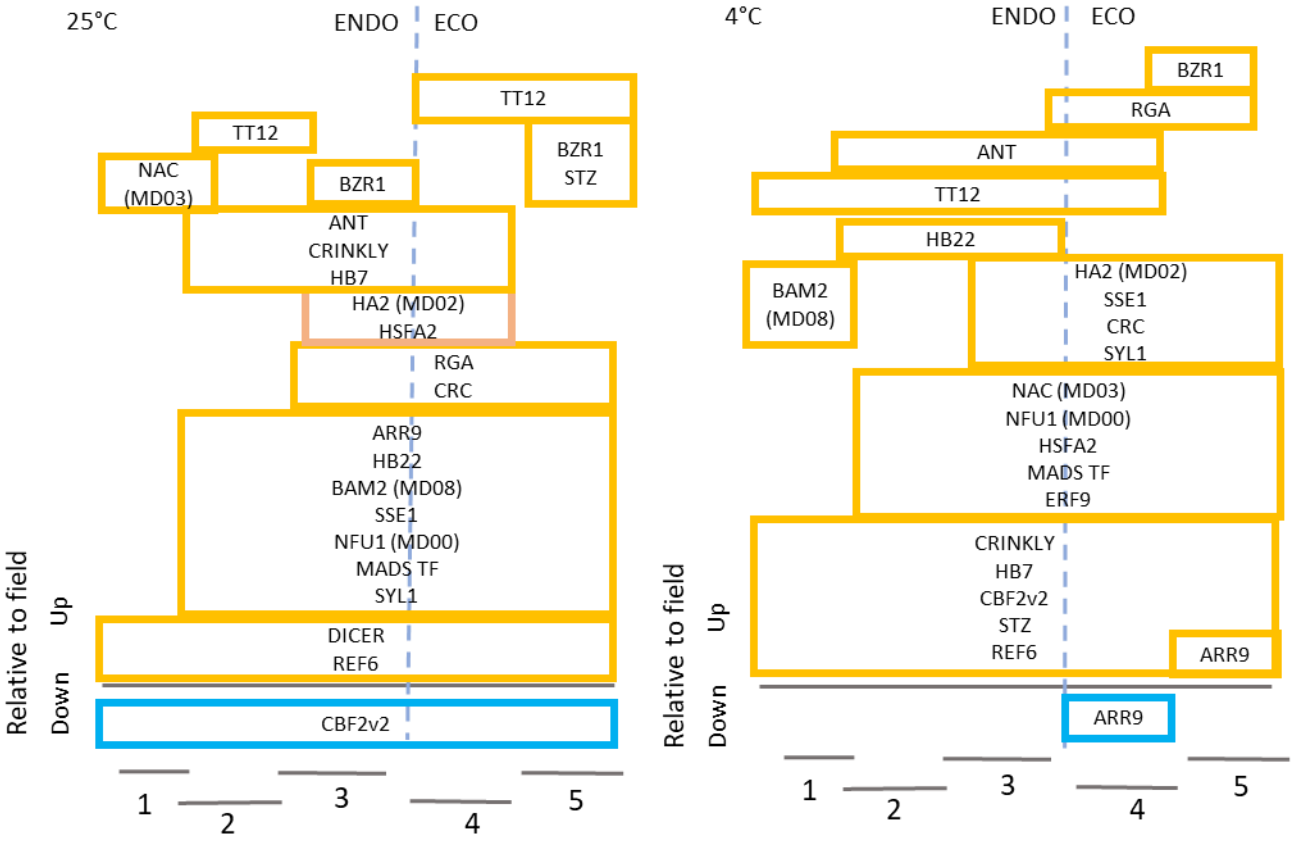


Figure 2-4 Simplified representation of expression profiles of candidate-genes in temperature test (25°C or 4°C) in comparison with same candidate-gene expression in the control samples from field. Numbered horizontal bars represent the time-points: three in endo- and two in eco-dormancy.

Hydrogen peroxide is a central redox signaling molecule and it is highly accumulated upon treatments with hydrogen cyanamide that induce bud break [26]. In our study, H_2O_2 treatments promoted bud break at particular moments of the endodormancy and ecodormancy phases of apple (Figure 2a). Similarly, H_2O_2

treatments in deciduous fruit crops, such as grapevine and pear, were reported to induce dormancy release and bud break [26,28], indicating that H_2O_2 could be part of the redox signaling that controls dormancy progression in fruit trees.

In order to shed light on possible genetic mechanisms underlying the H_2O_2-mediated bud break activation, the expression of dormancy marker genes and genes selected because their potential role in the dormancy cycle was quantified samples from H_2O_2 treated buds. In this experiment, none of the tested *DAM* genes showed differences in expression between H_2O_2-treated and untreated samples. This result is consistent with a previous report in which the expression of peach *DAM* genes was not altered seven days after treating buds with hydrogen cyanamide [29]. However, in another report, hydrogen cyanamide treatments led to the downregulation of the peach *DAM5* and *DAM6* two and four weeks after treatment, respectively [30]. These results suggest that the role of *DAM* genes in regulating ROS-mediated bud break induction is likely indirect and they are not part of the early responsive machinery. Remarkably, the H_2O_2 treatment promoted a down-regulation of *FLC*-like and an up-regulation of *CRINKLY* during the endodormancy (time-point 18/12/2020; Figure 2c). *FLC*-like is an apple homologous of the *A. thaliana* floral repressor *FLC*, which cold-mediated down-regulation is related to early flowering [3,5,31,32]. It has been shown that ROS and cold stabilize the protein VERNALIZATION 2 (VRN2), leading to its accumulation [23], which in *A. thaliana* acts as a repressor of FLC [33]. It is not known yet if this relation between ROS, cold, VRN2 and FLC is conserved between *A. thaliana* and Rosaceous species. In the presented experiment, *FLC*-like was specifically down-regulated by the H_2O_2 treatment during endodormancy (time-point 18/12/2020; Figure 2c, Annex I). During this period, *FLC*-like is still low expressed in buds until it is upregulated at the end of the endodormancy and towards ecodormancy [3,34] (Figure 2c, Annex I). Thus, ROS and cold signals might operate simultaneously to keep low *FLC*-like mRNA levels during the first steps of the endodormancy. The gene *CRINKLY* was also up-regulated in response to H202 at the same time-point as *FLC*-like (18/12/2020). *CRINKLY* encodes a transmembrane kinase receptor that has been mainly studied in *A. thaliana* roots, where it promotes formative pericycle cell divisions and restricts the number of pericycle cell divisions [35]. It is also known that CRINKLY is necessary for sepal development, ovule integuments and epidermis differentiation [36,37]. Its up-regulation

during endodormancy in response to H_2O_2 might thus be part of a developmental process linked to dormancy release and/or bud growth reactivation.

At the fourth time-point, the H_2O_2 treatment led to the up-regulation of the *NFU1* iron-sulfur cluster protein (source: Araport11) and the down-regulation of *GRP6*, which is the homolog of the *A. thaliana* gene *GA-STIMULATED ARABIDOPSIS 6* (*GASA6*) (Figures 2c, annex I). This latter gene acts as a hub in GA and BR signaling. It is down-regulated by ABA and is highly expressed during seed germination of *A. thaliana* [38,39]. Interestingly, *GASA6* overexpression in *A. thaliana* attenuates the negative effect of ABA during seed germination [40]. This information does not directly match with the idea that this gene could be involved in the dormancy cycle

The study of the effect of H_2O_2 on bud break and the expression of dormancy-related genes enables us to hypothesize that ROS signaling plays a role during the dormancy cycle by acting on GRNs controlling dormancy in apple [3,5]. Further studies will be necessary to unravel the role of studied genes (e.g., *FLC*-like, *GRP6*, etc.) on the phenological and molecular responses we observed after this test of oxidative stress application.

4.2 *SVPa* is potentially involved in the response to temperature modulation during endodormancy release in association with other genes besides the *DAM* genes

The group of genes associated with dormancy and called markers-genes in this study had their expression evaluated in the temperature and redox tests for a better understanding of their roles during the endodormancy and ecodormancy stages and transitions. As previously said, no significant differences were observed in this group of genes in the H_2O_2 treatment. In the case of temperature tests, significant gene expressions were observed. Most of the *DAMs* genes (*DAM2*, *4* and *b*) were repressed when buds were submitted to heat (at 25 °C) close to the stage corresponding to CR achievement in Golden delicious (800 CH). Moreover, *CBF4,* known as a cold-induced gene, presented a response to both cold and warm temperatures. It was up-regulated after the 4 °C treatment and down-regulated after the 25 °C treatment. Those results are consistent with previous literature [41]. Indeed, the down-regulation of *DAM2* and *DAM4* just before the endodormancy release, associated with constant levels of *SVPa* at the same time, fits the putative model recently proposed based on SVPa dimmers

[42]. In this putative model, DAM, FLC-like and SVP-like TFs form dimers. Different types of dimer combinations are formed corresponding to different genes that are activated/repressed during dormancy progression in association with the environmental changes (e. g. cold). Regarding the 4 °C treatment, *CBF4* up-regulation was observed but not *DAM1* regulation, although this gene is known to be induced by CBF4 in [10,43]. As gene expression was measured 24 h after treatment, it's possible that this delay was too short to activate *MdDAM1*.

It is noticeable that *SVPa* was induced by both treatments, either 4 °C or 25 °C treatment, and also at the same time point. Speculatively, the up-regulation of *SVPa* after 4 °C treatment and at the endodormancy release stage, fits the dimers formation of SVPa with a growth repressor or a DAM TF. However, the transcriptional induction of *SVPa* at 25 °C, the temperature at which its protein partners might be underrepresented (Figure 3), could indicate a role of SVPa at warm temperatures during dormancy. This role could be part of a molecular mechanism to prevent endodormancy release by short heat periods (or be part of a different developmental process), and if SVPa interacts with other TFs at warm temperatures remains unclear yet.

4.3 There are potentially two distinct types of machinery that preserve bud dormancy: one in endodormancy and another one in ecodormancy

The gene *STZ* is widely known for being part of the signaling cascade that responds to cold and is involved in the adaptation to cold and other abiotic factors [44]. This gene is up-regulated at all the time-points after the 4 °C treatment, a result that matches the bibliography [44]. However, more surprisingly, it is also up-regulated after the 25 °C treatment in the last time point. A possible explanation for the observation at 25 °C could be that *STZ* would be activated by the ROS signaling during endodormancy once cold and other stressing factors (such as salinity or pathogens) are not present. As no difference was observed in *STZ* expression between treated and control in the H_2O_2 test, this explanation is unlikely and will require further studies. Other genes, *ERF9*, *NAC* and *CBF2v2,* were up-regulated in response to cold treatment, independently of the stage of dormancy. These genes are known to be transcriptionally activated by desiccation, caused by cold and salinity [45–47], thus

potentially blocking growth. Therefore, their up-regulation by cold treatment at most of the time-points could be part of a mechanism of tree adaptation to unfavorable environmental conditions. Notably, the *NAC* gene is a homolog of the *A. thaliana* drought-induced transcription factor *RD26* (*RESPONSIVE TO DESICCATION 26*), which is believed to inhibit brassinosteroid-regulated plant growth [48]. Our results thus suggest that *NAC,* and maybe *ERF9 and CBFv2,* act as a dormancy inducer (or growth inhibitor) and contribute to ensuring bud dormancy when environmental conditions are not yet suitable for proper development. The up-regulation of *RGA-like2* could imply its function as a growth repressor (Figure 4). *RGA-like2* encodes one DELLA protein that negatively regulates the seed germination in *A. thaliana* by acting as a negative regulator in the GA signaling pathway [49]. In addition, in apricot and peach was shown the potential regulation of endodormancy release by GA through *RGA-like2* regulation [50,51]. *Populus* trees with lower levels of active GAs due to lowered perception by overexpression of *GA INSENSITIVE* (*GAI*) or *RGA-like* genes showed early bud set and late bud burst [52]. Bioactive gibberellin levels are correlated with endodormancy release and bud break in fruit trees [50]. The sudden change of temperature to 4 °C on samples collected in field conditions during ecodormancy could have triggered *RGA-like2* expression to counteract GA signaling and kept growth inhibition during the ecodormancy state. Interestingly, *RGA* was up-regulated 24 h after the 25 °C treatment yet in the endodormancy stage (time-point 01/11/2020; Figure 4, annex II). Therefore, *RGA-like2* transcriptional activation at 25 °C could be part of a mechanism to block gibberellin signaling and avoid growth during endodormancy as it happens in ecodormancy in apricot and peach.

4.4 The process of endodormancy release is gradual and might start at earlier stages during the dormancy cycle

The apple homolog of the *A. thaliana type A RESPONSE REGULATOR* (*ARR*) *ARR9,* which is part of cytokinin signaling, was upregulated from mid-endodormancy to ecodormancy after 25 °C treatment. This finding matches the description of type-B (BRR) in apple and their putative influence on endodormancy release [38]. Type-B ARRs regulate the transcriptional output from the CK pathway, while type-A ARRs act as negative feedback regulators to CK desensitizing [53]. Three *MdBRRs* were specially induced to warm temperatures when the CR was fulfilled. Yet the down-

regulation of *ARR9* just after the endodormancy release after application of cold temperature (4 °C treatment) indicates that cytokinin and possibly other *ARR/BRR*s could be involved in growth regulation and ecodormancy maintenance. Another growth-related gene, *BARELY ANY MERISTEM 2* (*BAM2*), a leucine-rich repeat receptor-like serine protein, is known for being related to floral meristem fate [39,41] was also up-regulated at 25 °C around the endodormancy release time point. The *endoribonuclease Dicer 3* (*DICER3)* was found up-regulated at 25 °C from mid-endodormancy until ecodormancy. Potentially, DICER3 and the RdDM are acting in the epigenetic regulation by DNA methylation of an important group of genes [42,43]. This regulation could contribute to the endodormancy release and start of heat accumulation leading to bud burst and flowering. Previous findings in *A. thaliana* suggest an important role of *DICER3* as a player of RdDM (with other genes and miRNAs) in the chromatin modification and *FLC* silencing, which leads to flowering [43]. It has been reported a role of DICER3 in the RdDM-mediated regulation of dormancy (seed and bud) and flowering [42,43]. Warm temperatures induce *DICER3* in a way that could lead to DNA methylation and/or RNA interference silencing of dormancy-related genes (e.g., *FLC*-like), thus promoting (with probably other actors) endodormancy release and bud break/flowering even below the CR. If such a pathway exists, this could be the way that bud break occurs in warm winter, creating uneven and erratic bud break and poor flower production and production.

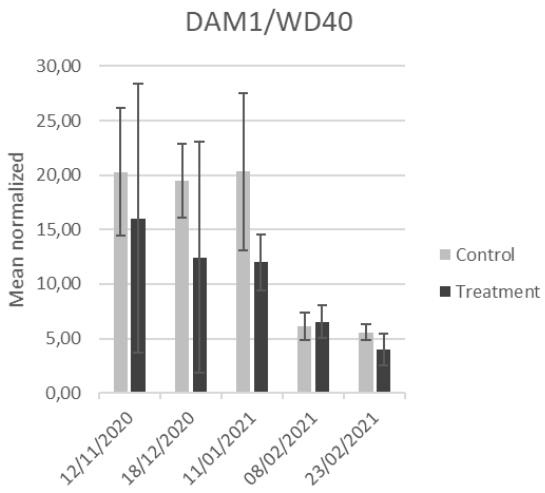
Several genes whose expression has been quantified in this section showed changes in expression in response to temperature at distinct dormancy phases. The strong response of genes to 25 °C treatment can be considered a rapid response to temperature stress. Indeed, the average temperature in January was around 8 °C, and a change to 4 °C for the controlled condition experiment had less impact than a change to 25 °C. Therefore, the genes related to growth, epigenetic regulation and hormonal signaling for which changes in expression were observed in 24 h treated samples could be responding to environmental stress to achieve acclimation. This rapid response could also be part of developmental machinery that operates already during the winter dormancy to establish an intermediate dormancy status between endodormancy and ecodormancy. This could be mediated by altering the epigenetic landscape of dormancy- and growth-related genes to combine acclimation and developmental responses simultaneously during a progressive switch from endo- to eco-dormancy.

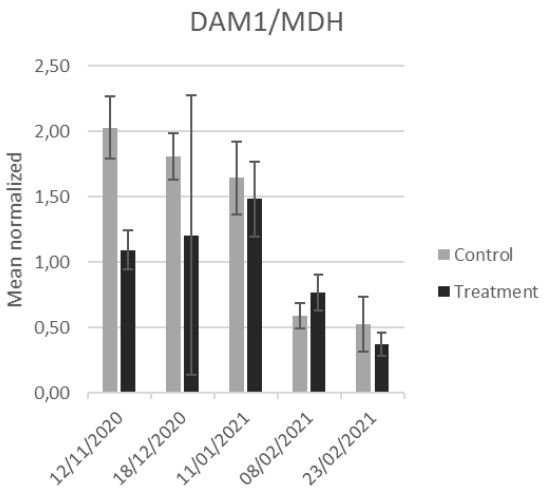
5 Conclusion

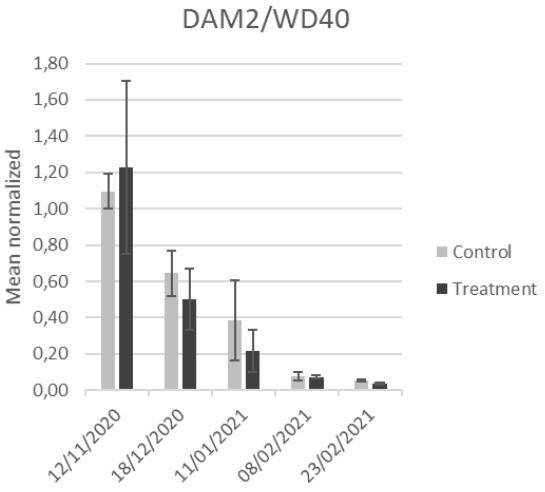
Redox signaling was shown to be effective in activating bud growth in apple even during endodormancy. A potential link between ROS and gene expression in the context of endodormancy release and bud break was also found. Moreover, in this work, we could discriminate genes that respond to temperature depending on the dormancy stage. Our results suggest a possible genetic regulation of the bud dormancy that potentially also involves the dormancy-associated genes. However, further studies are required to fully understand the role of each gene and decipher possible GRN involved in the temperature-mediated control of dormancy in fruit trees. In this study, we suggest that a combination of redox signaling, acclimation and developmental responses to temperature could be part of the genetic (and epigenetic) regulation of dormancy, in addition to the already demonstrated hormonal and transcriptional regulations. We thus open new perspectives to understand bud dormancy and prepare new plant material better adapted to the near future under climatic changes.

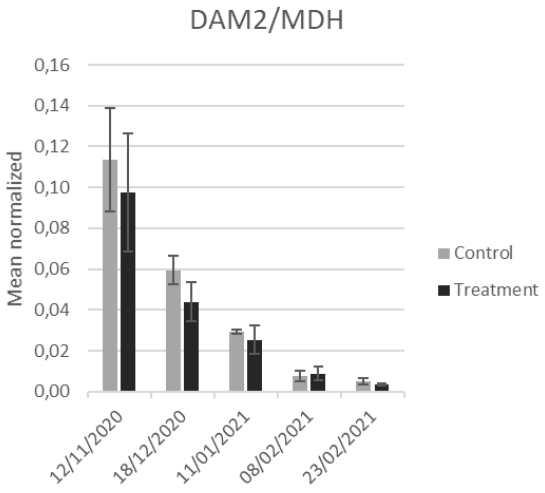
ANNEX I

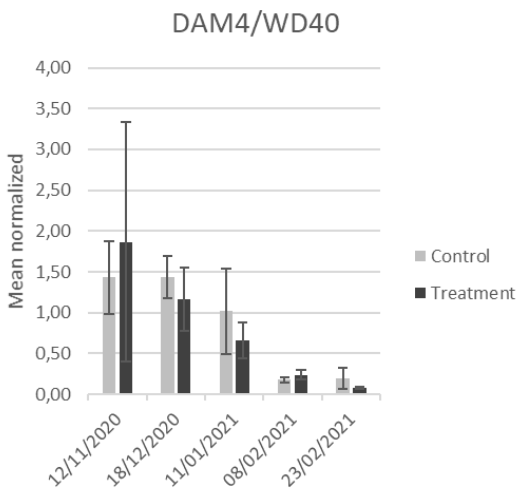
RT-qPCR of Dormancy Marker Genes and Candidate Genes on control and treated samples of REDOX test

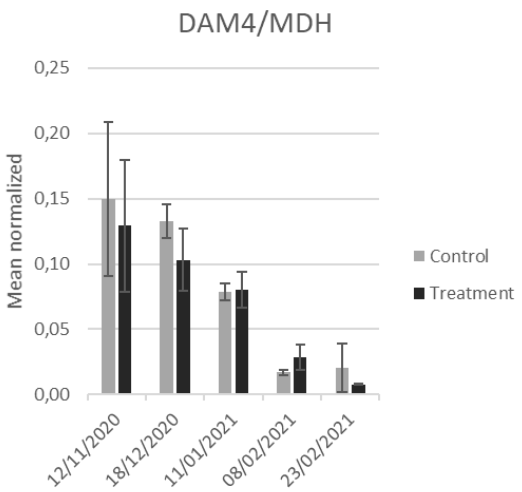






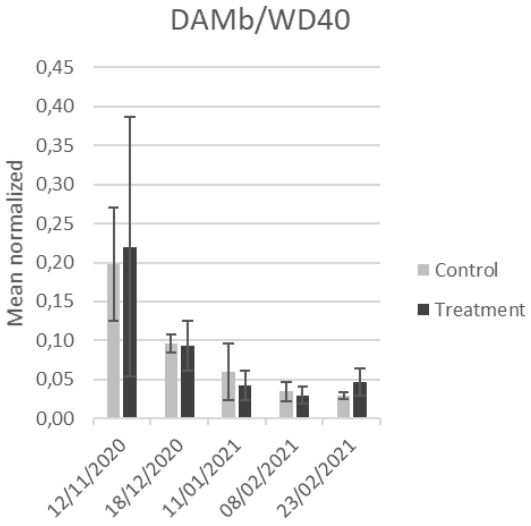


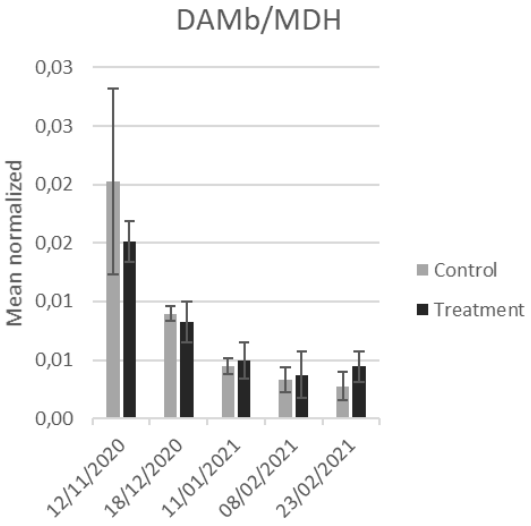


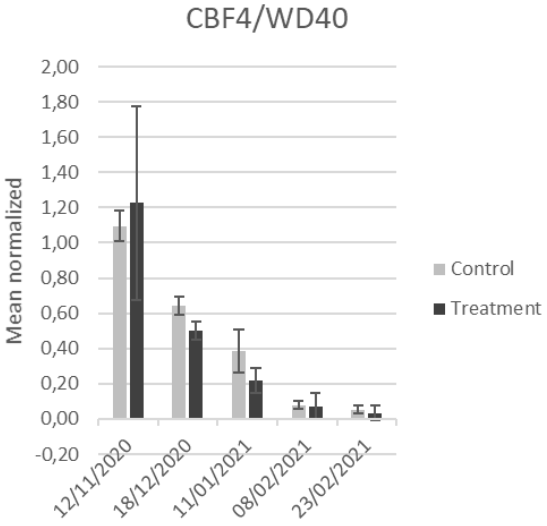


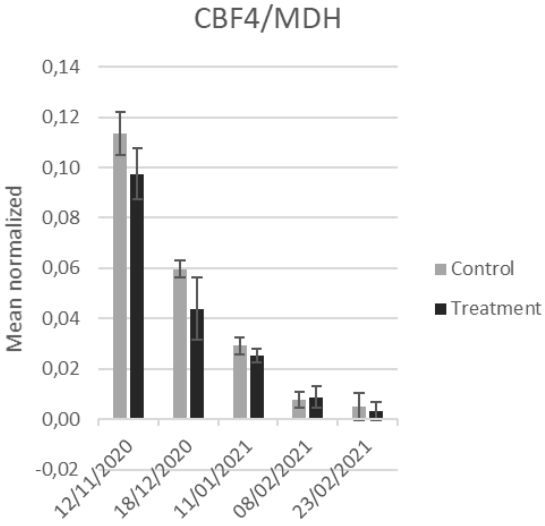
Annex I

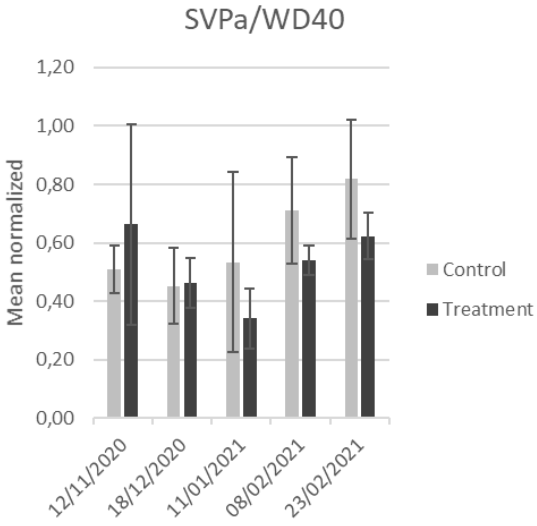
Dormancy marker genes

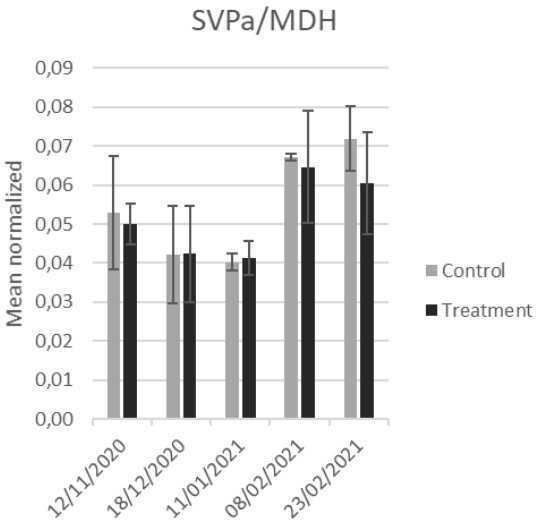








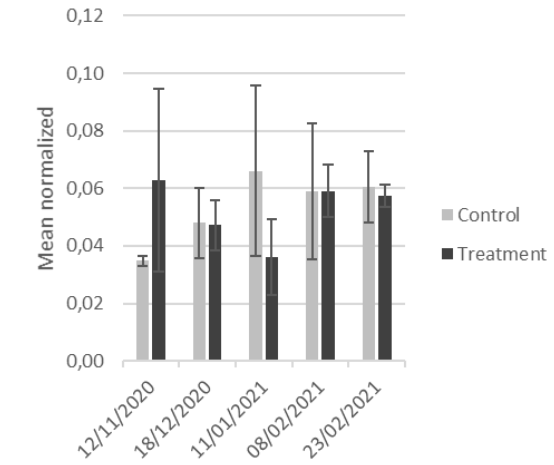




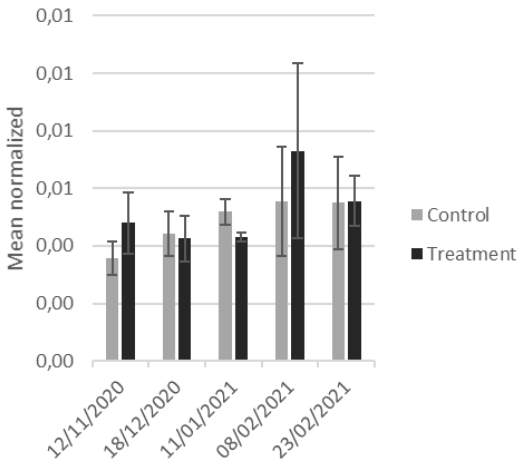
Annex I

Dormancy marker genes

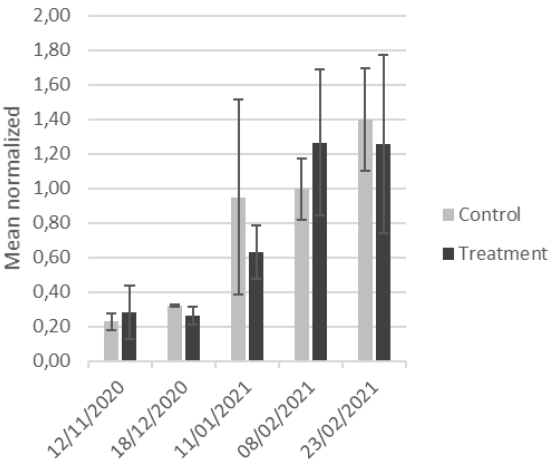
SVPb/WD40



SVPb/MDH



MAF2/WD40



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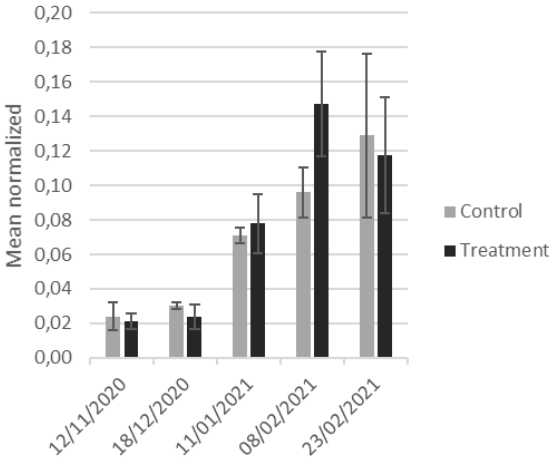
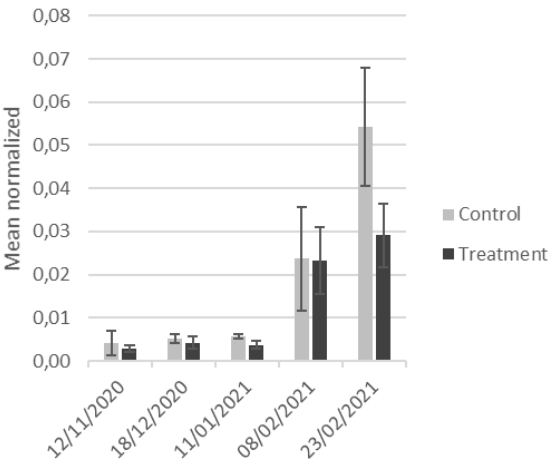


EBB1/WD40



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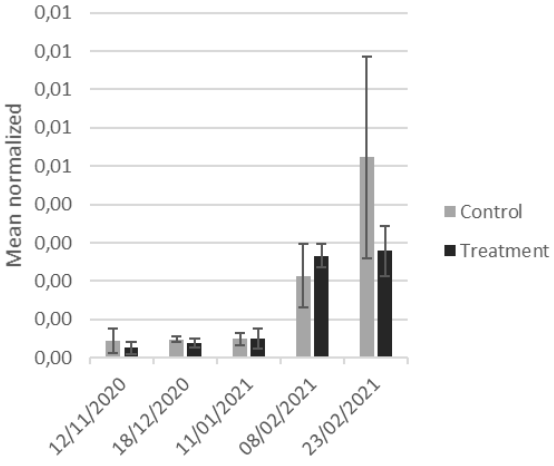
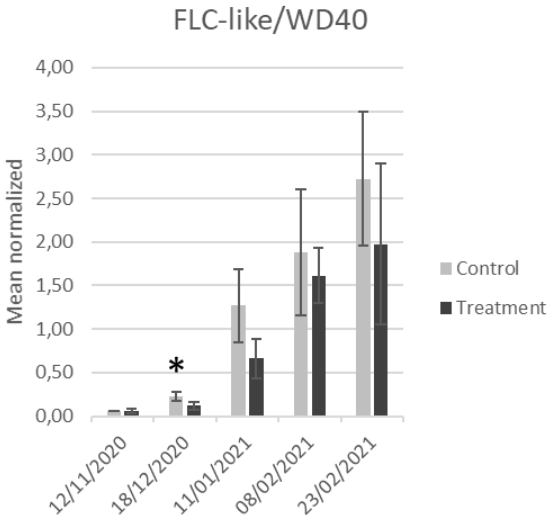
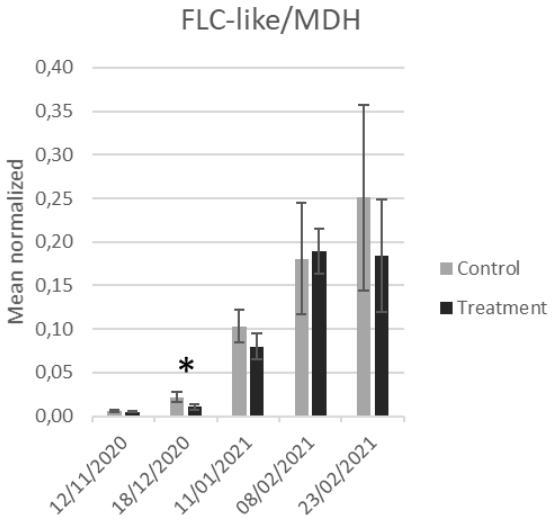


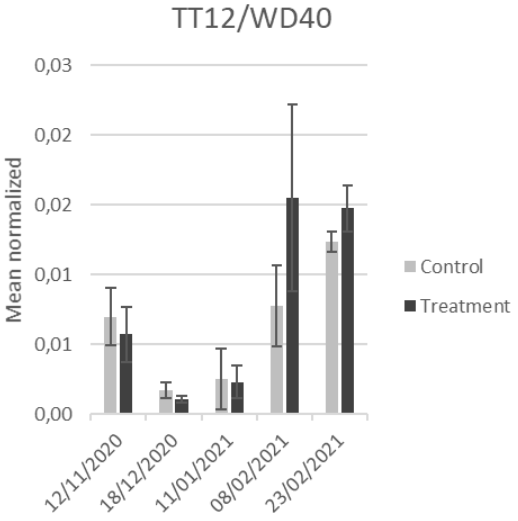
Annex I

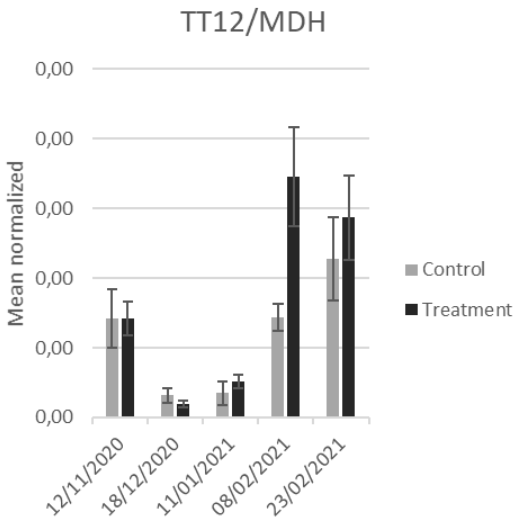
Dormancy marker genes

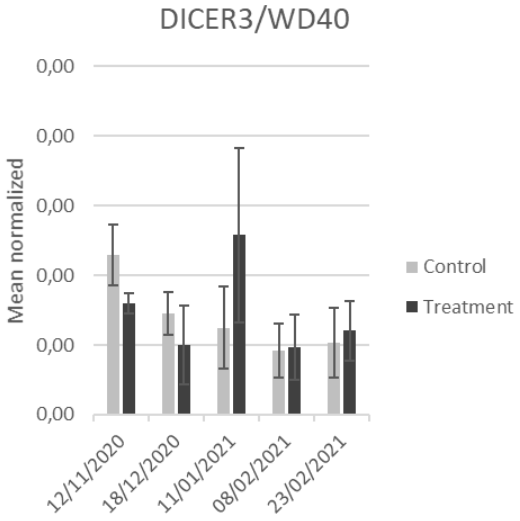


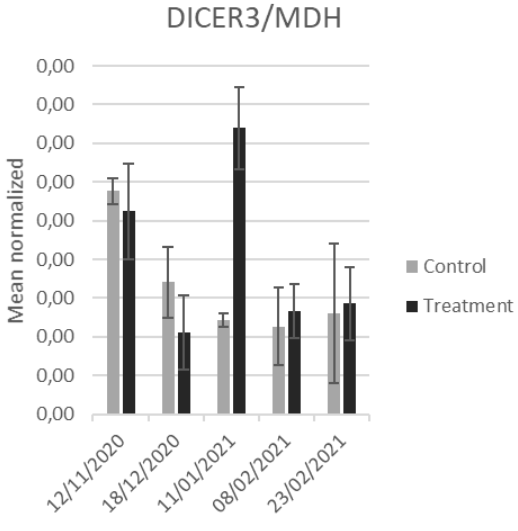


Candidate genes





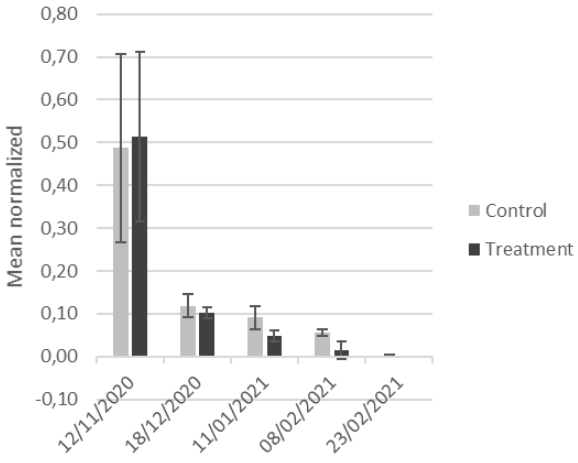




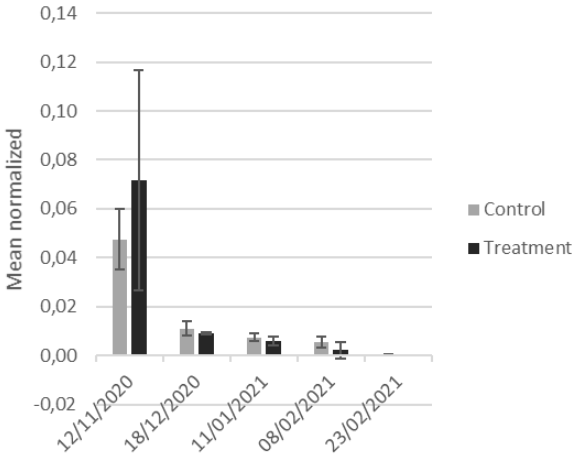
Annex I

Candidate genes

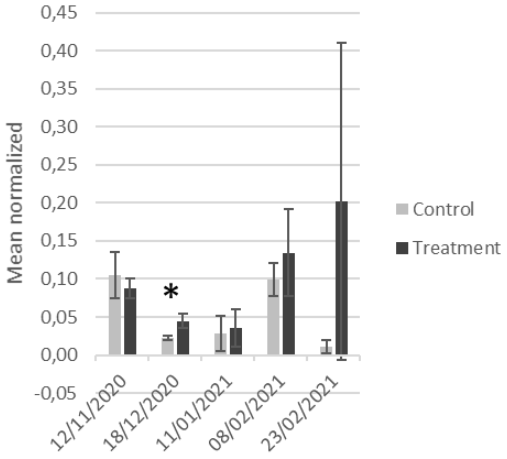
CBF2v2/WD40



CBF2v2/MDH



CRINKLY/WD40



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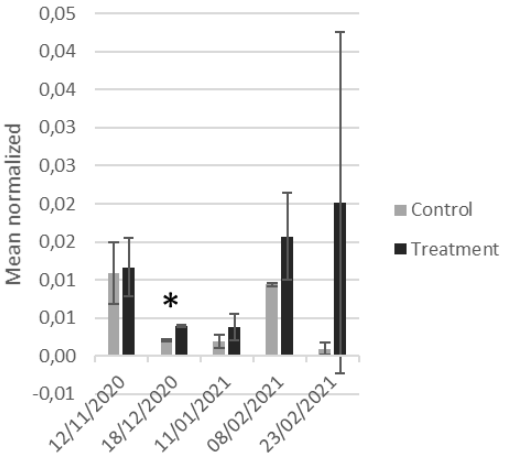
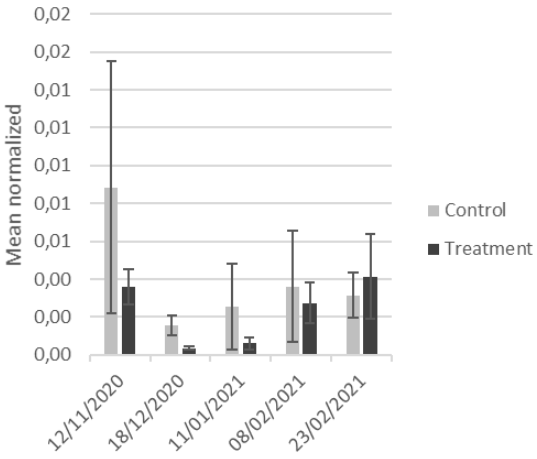


ERF9/WD40



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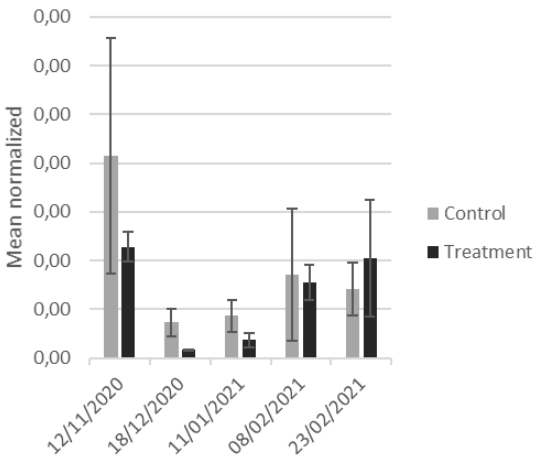
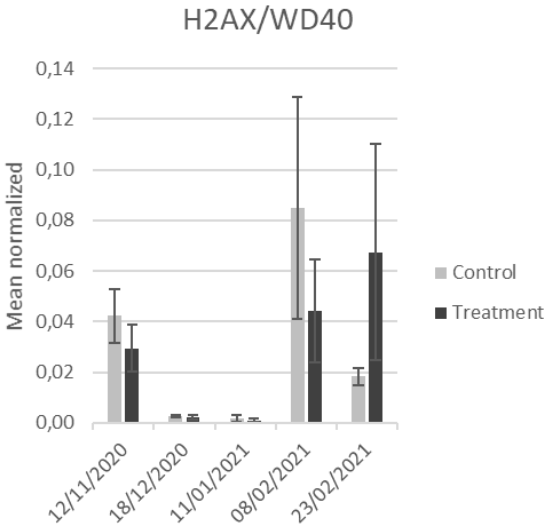
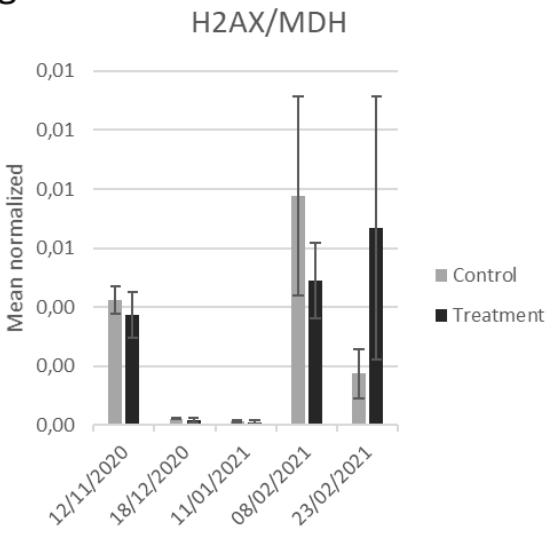
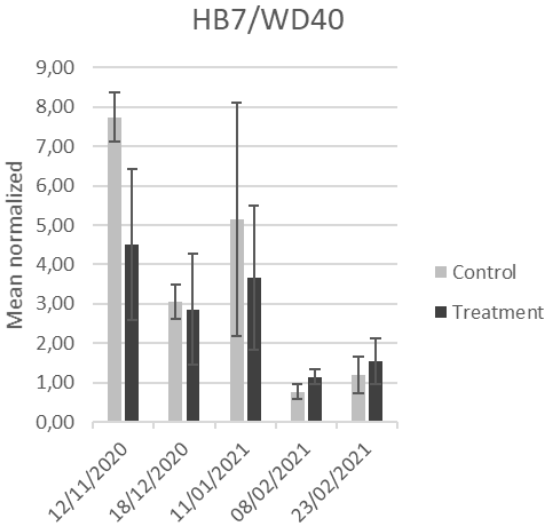


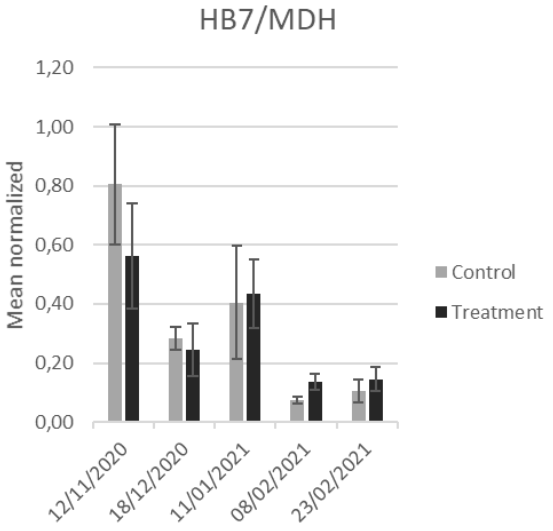
Annex I

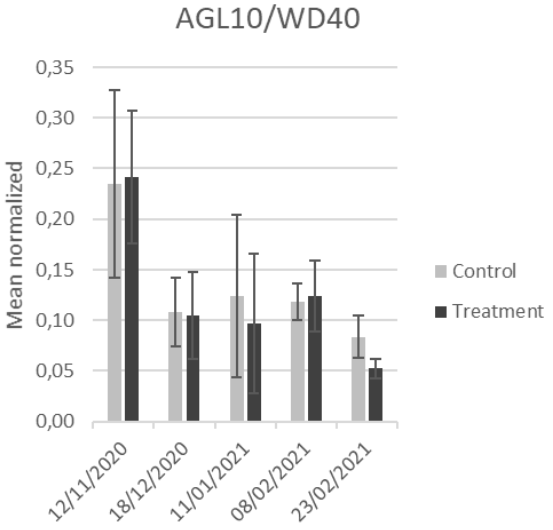
Candidate genes

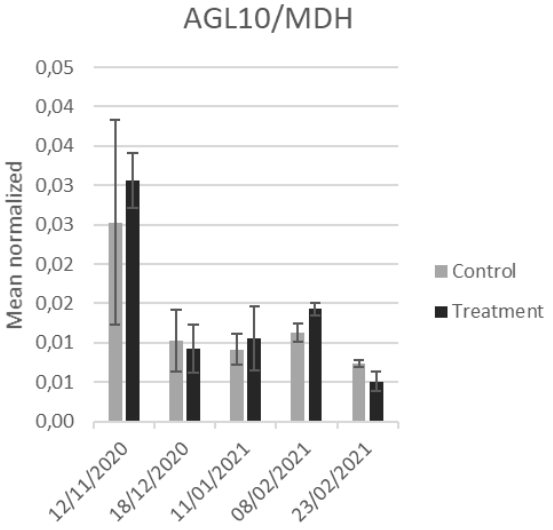








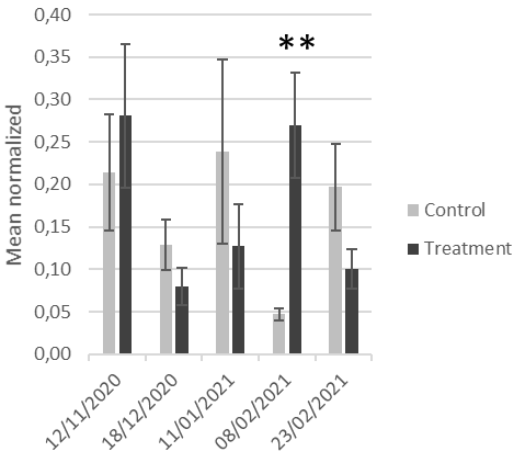




Annex I

Candidate genes

NFU1/WD40



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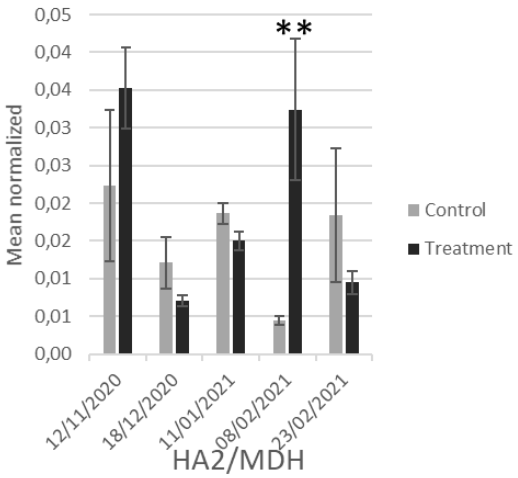

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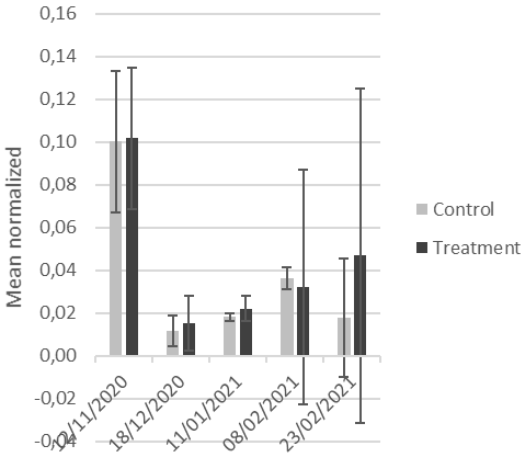

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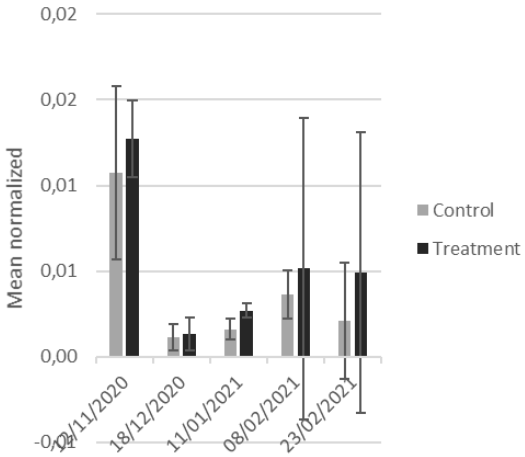

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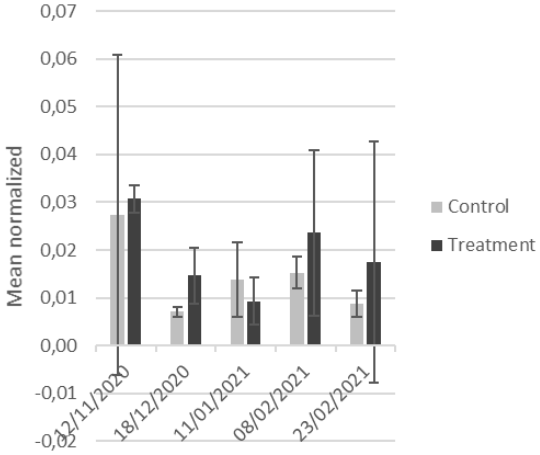

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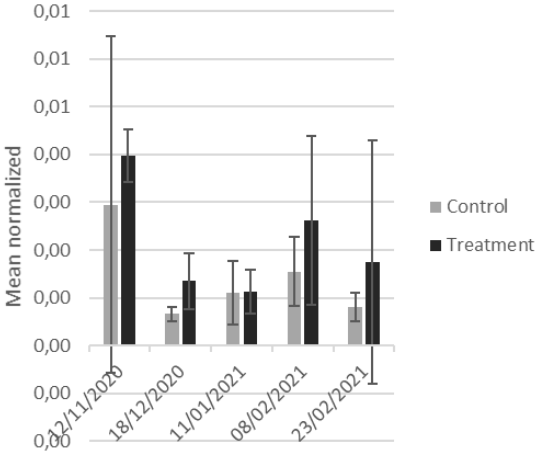
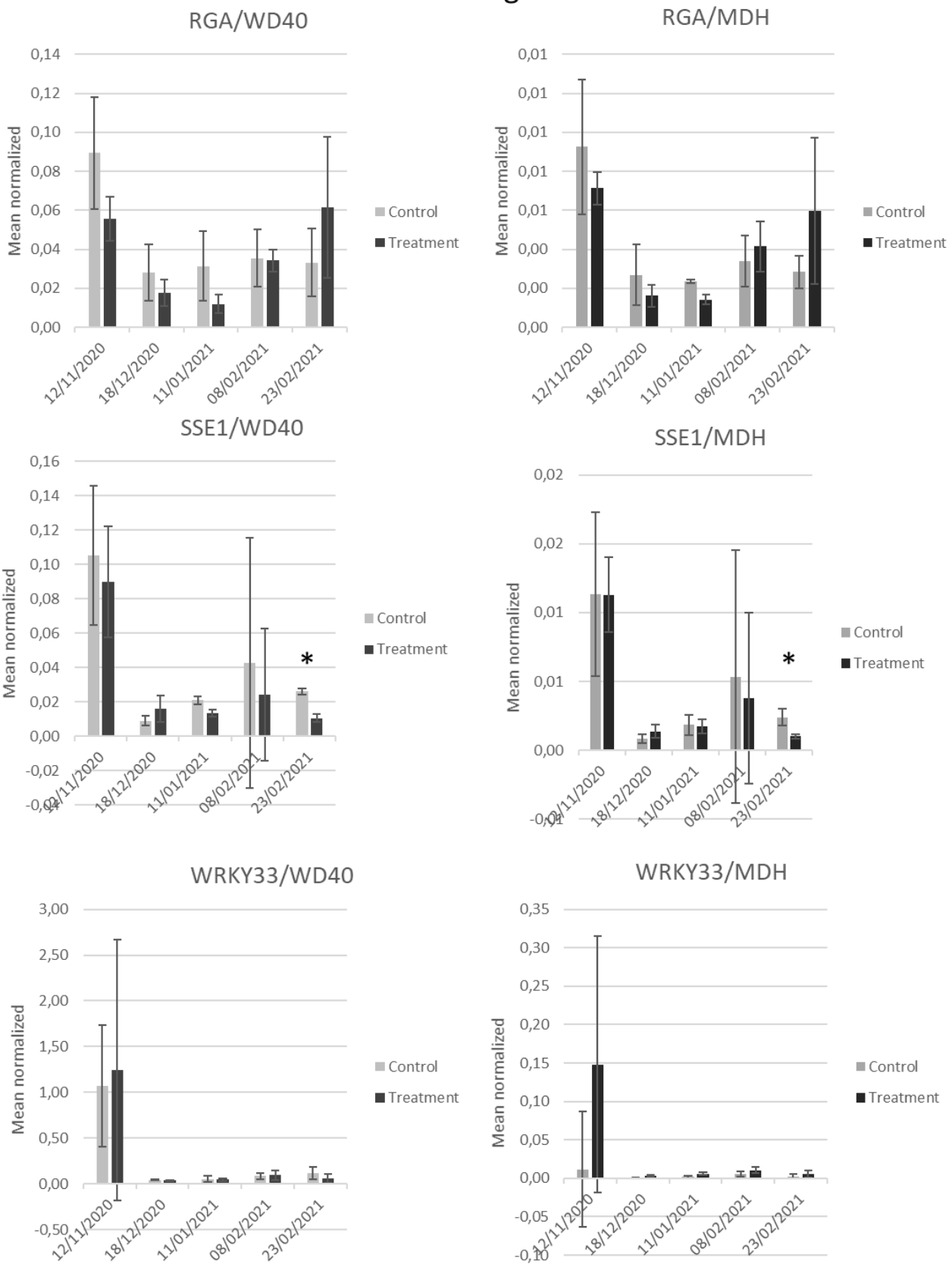


Annex I

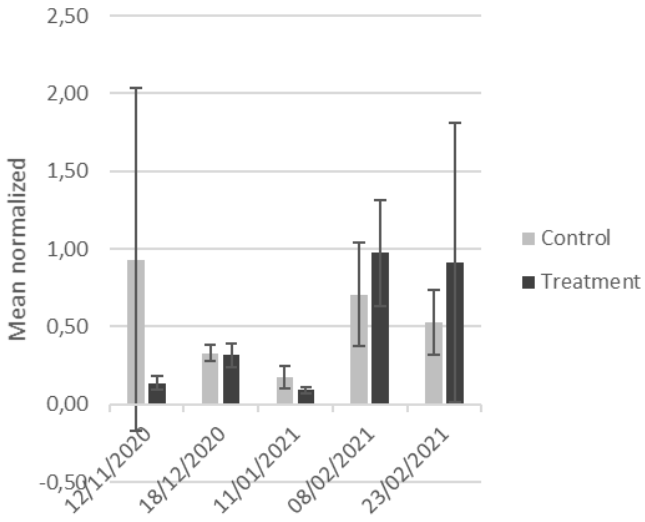
Candidate genes



Annex I

Candidate genes

STZ/WD40



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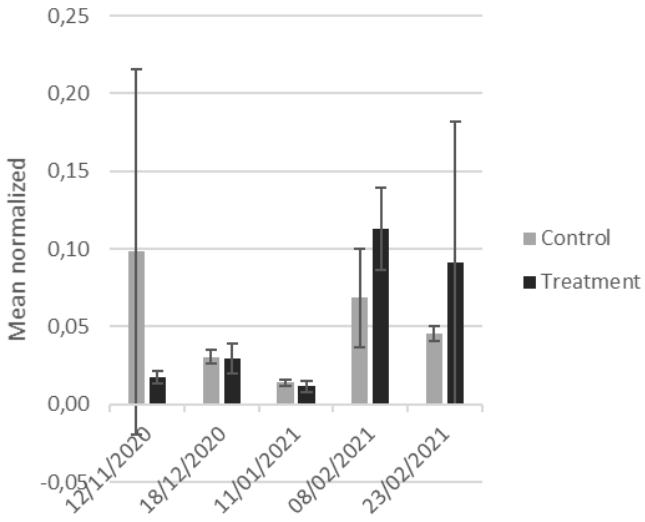

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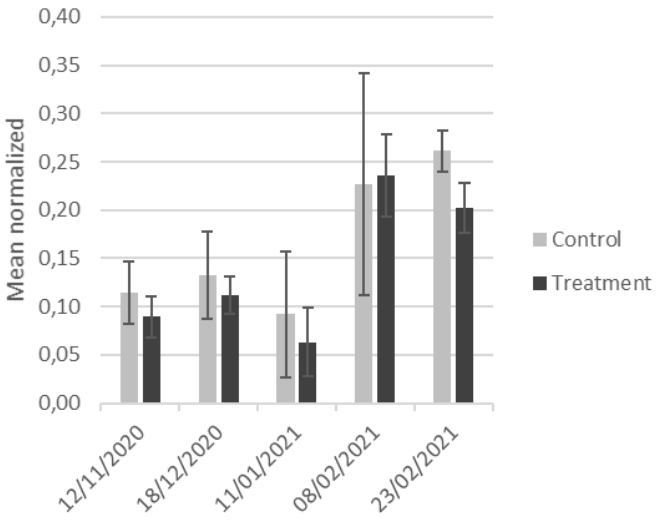

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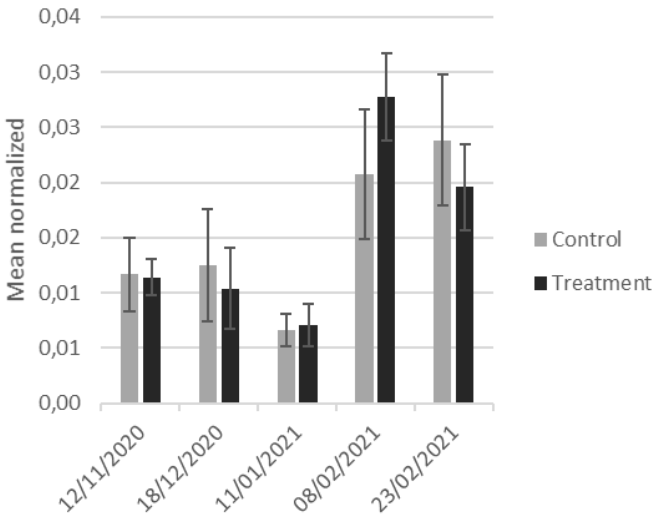

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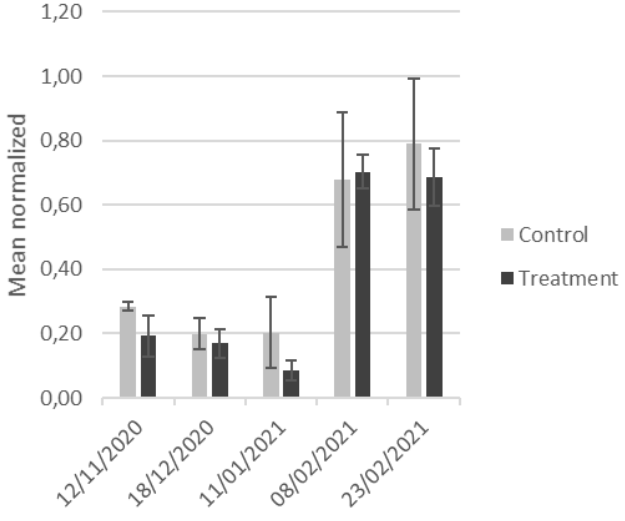

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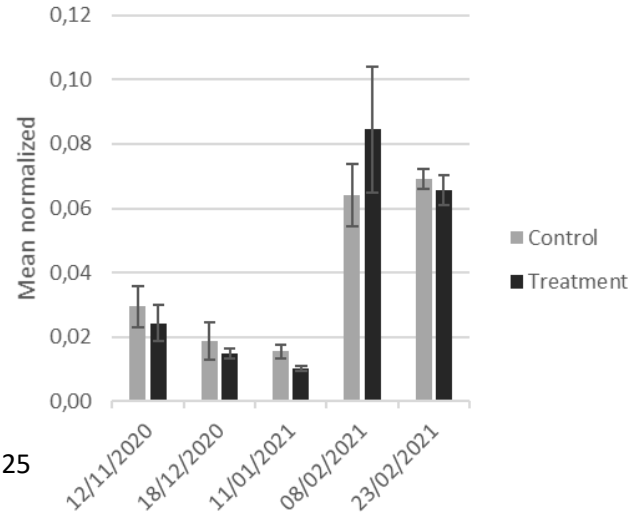
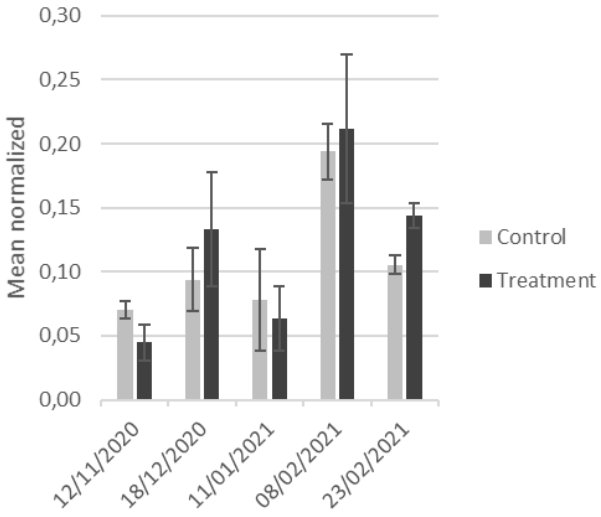


Annex I

Candidate genes

REF6/WD40



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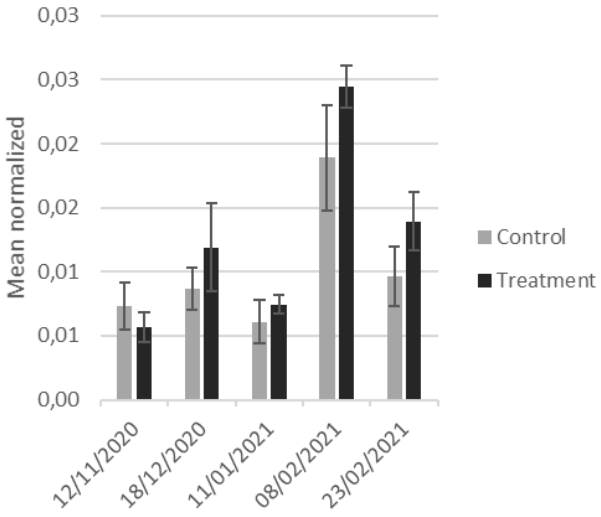

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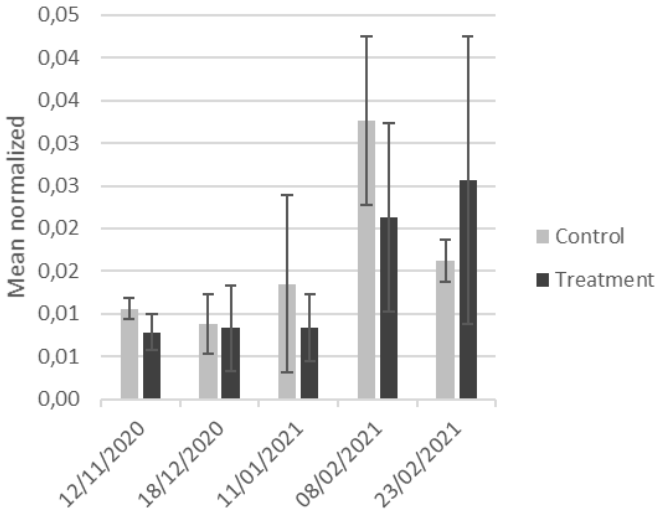

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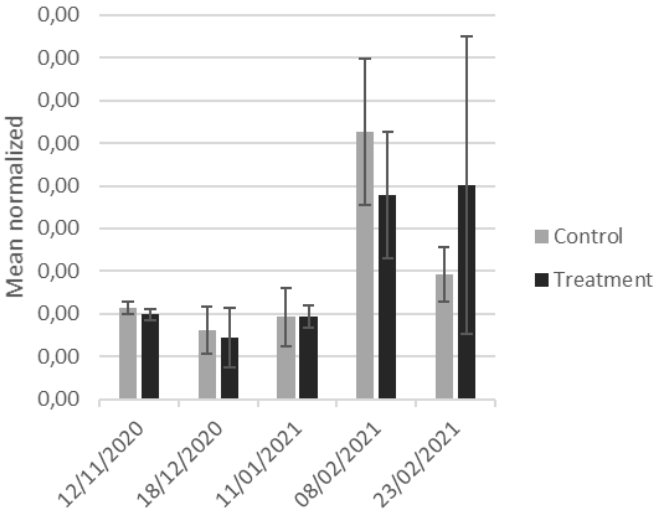

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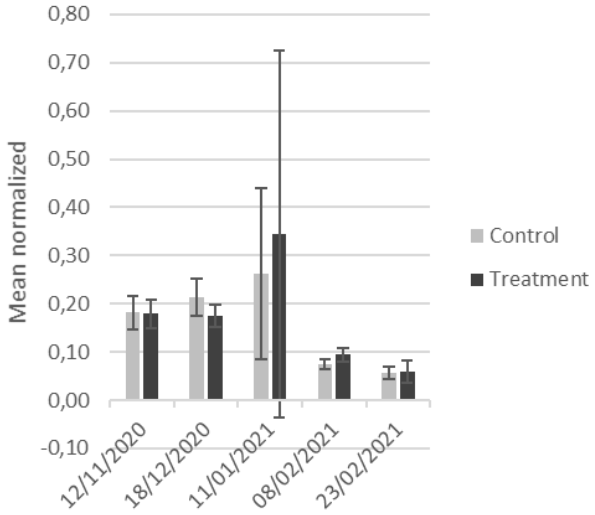

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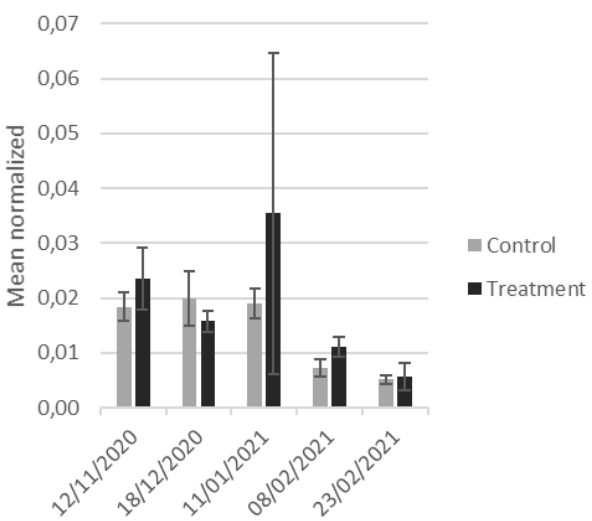
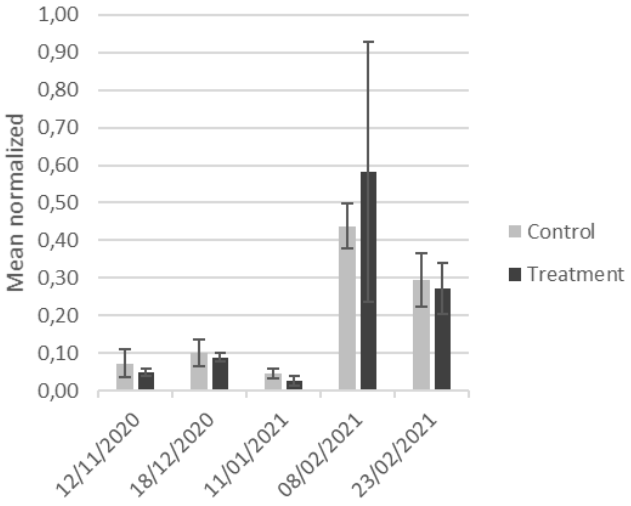


Annex I

Candidate genes

HB22/WD40



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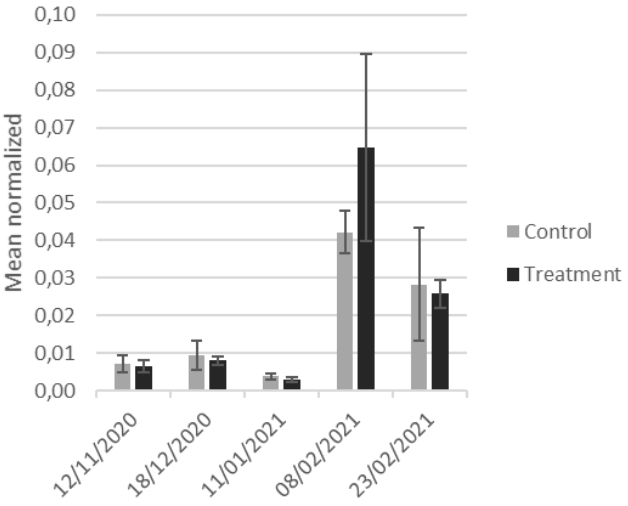

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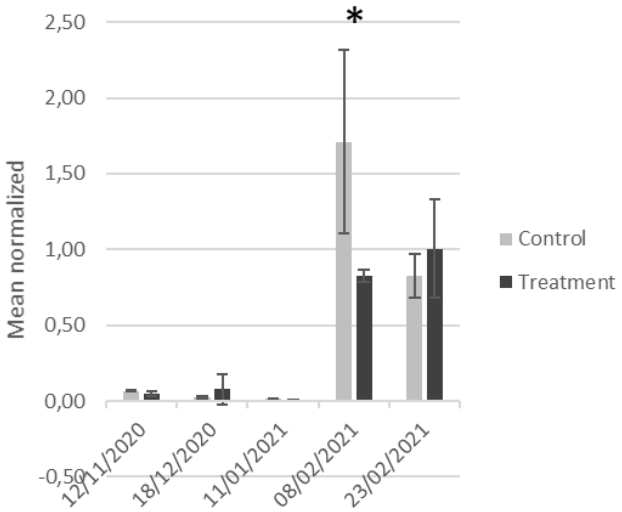

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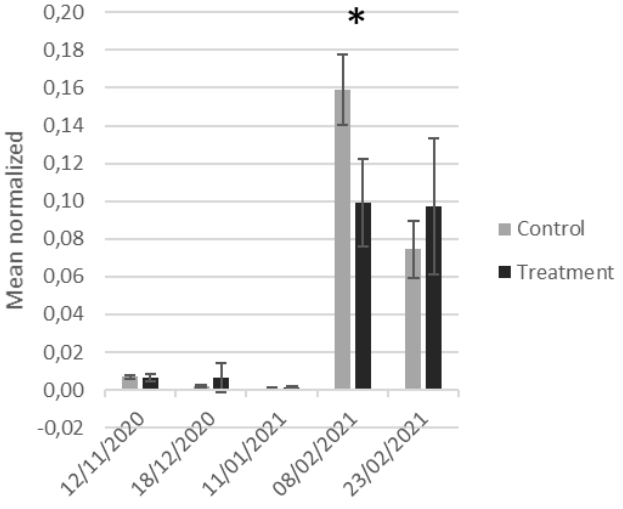

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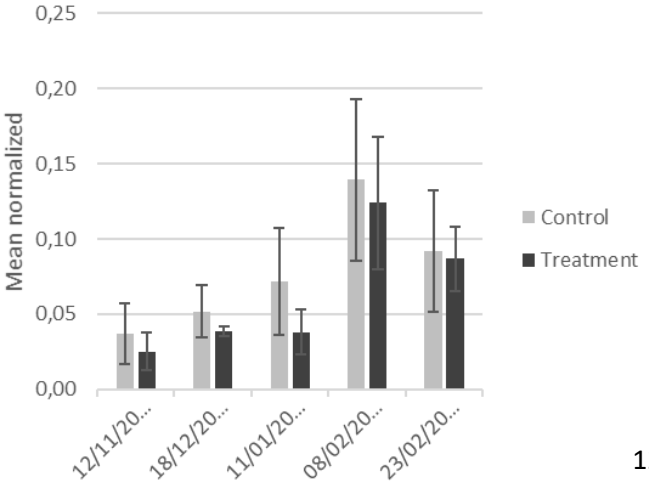

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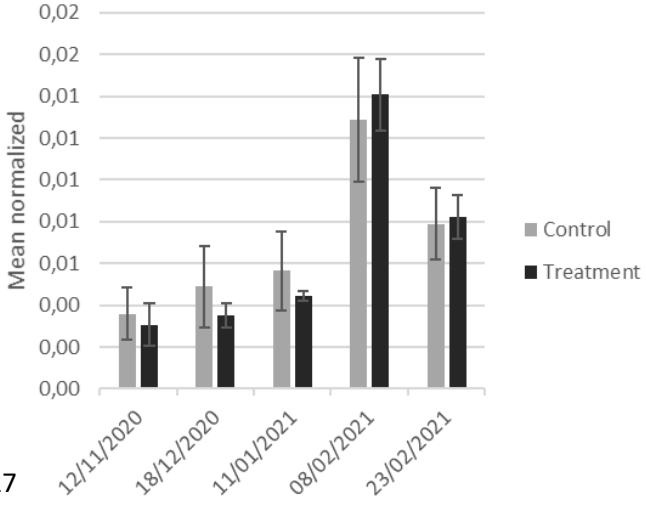
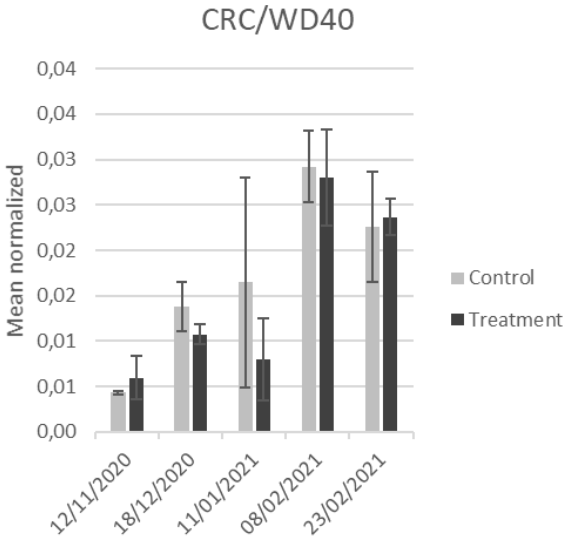
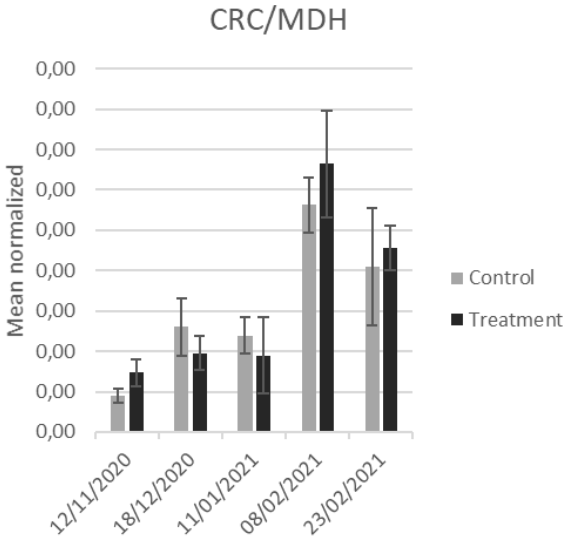
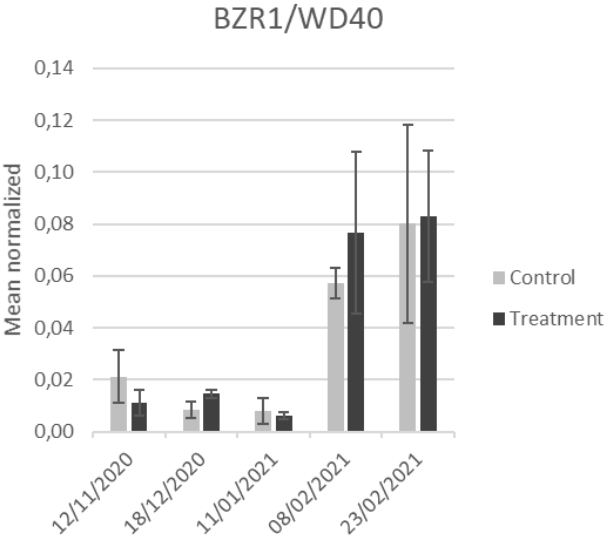


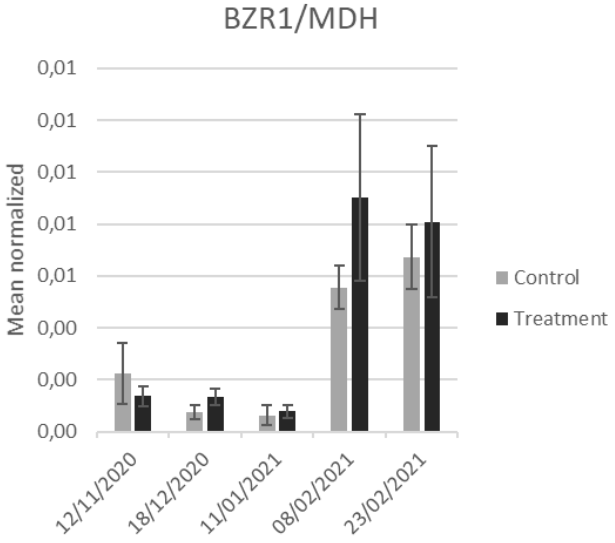
Annex I

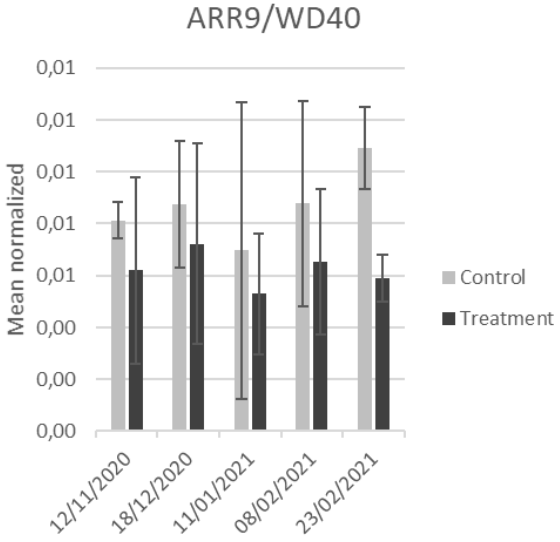
Candidate genes

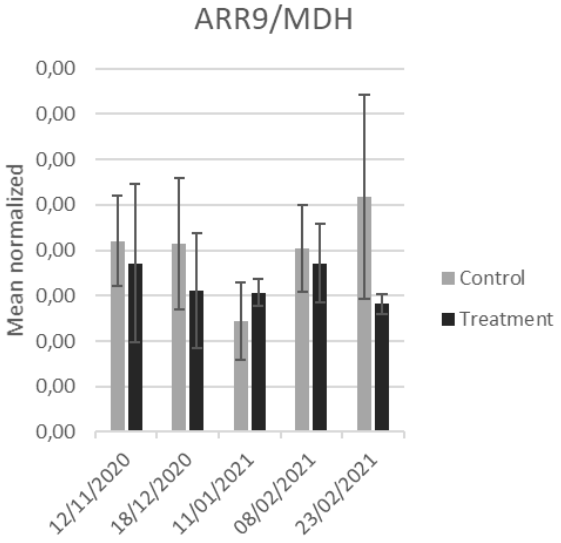






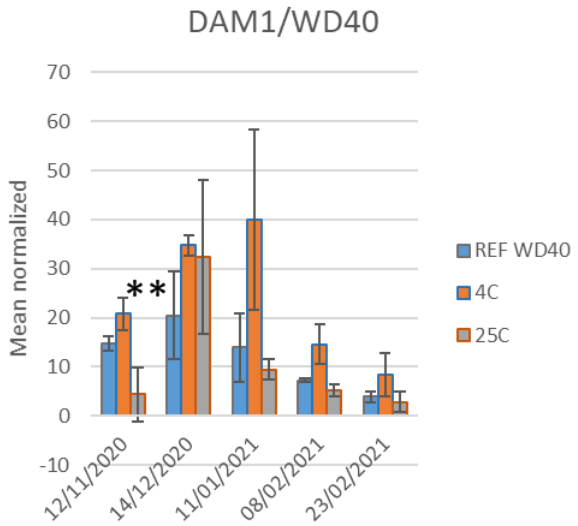


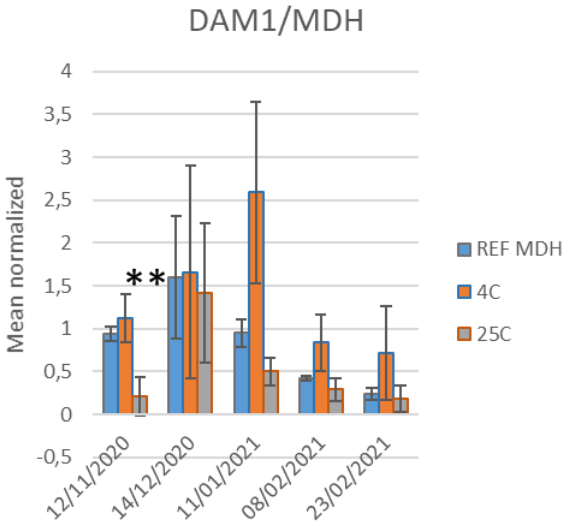


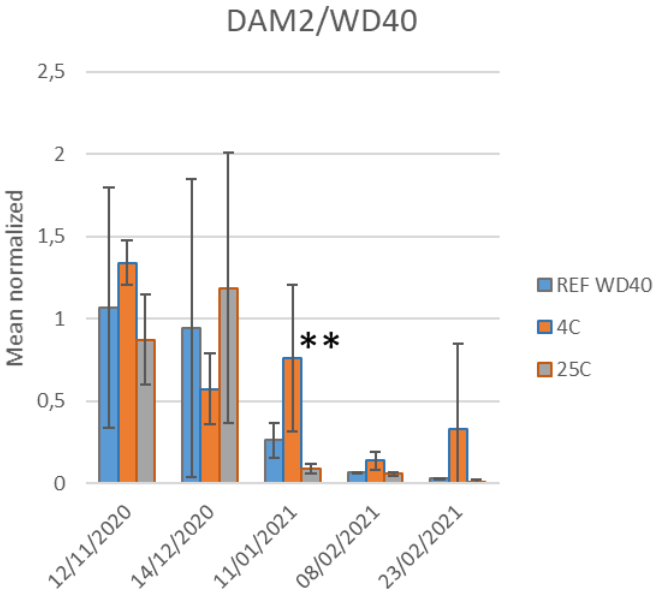


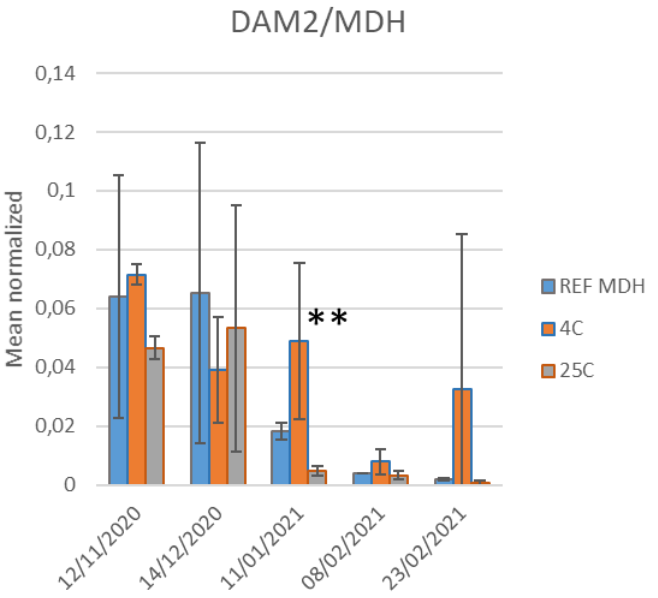
Annex II

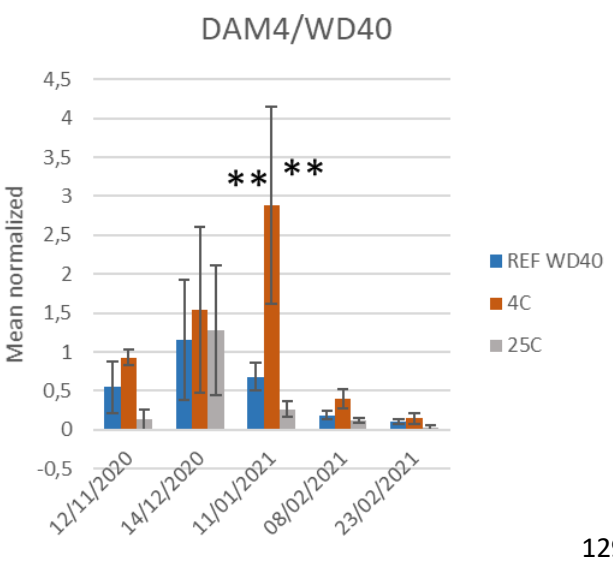
Dormancy marker genes

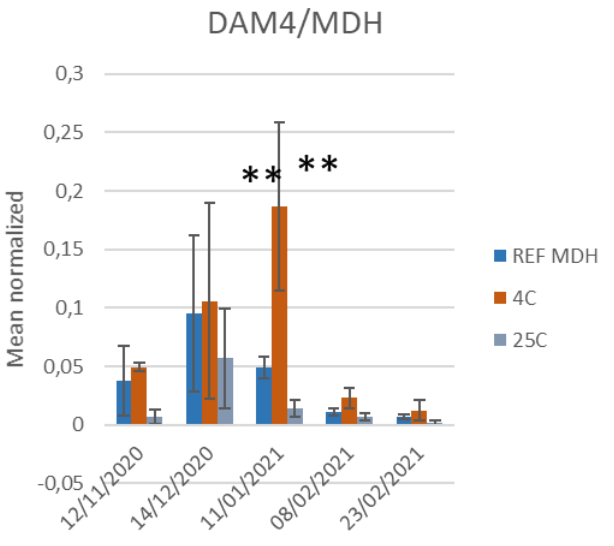








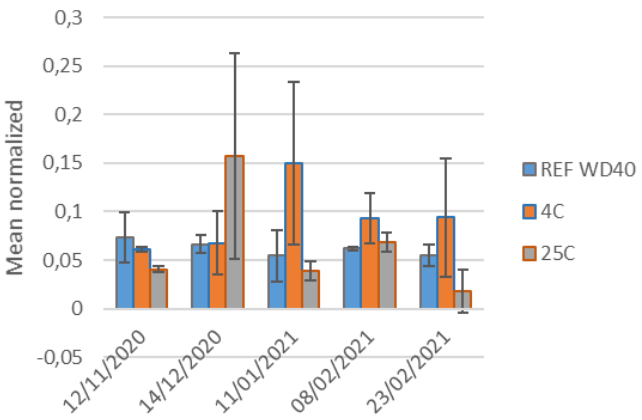




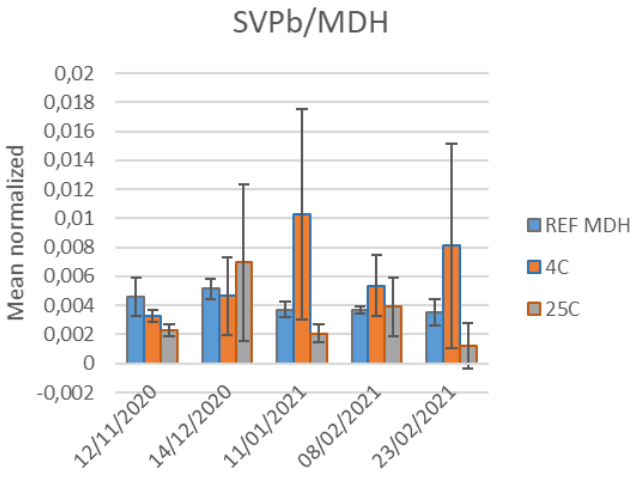
Annex II

Dormancy marker genes

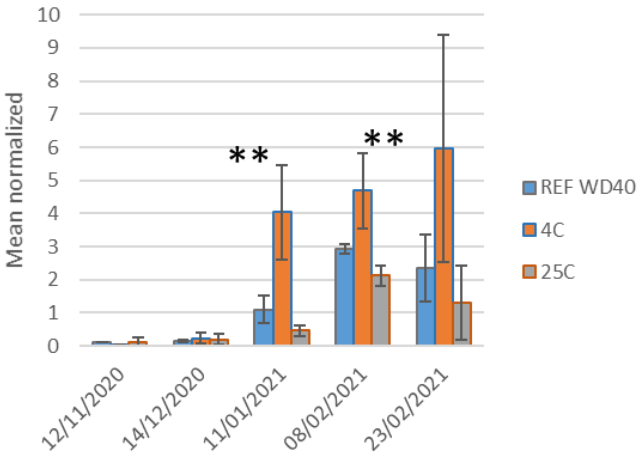
SVPb/WD40



SVPb/MDH



FLC/WD40



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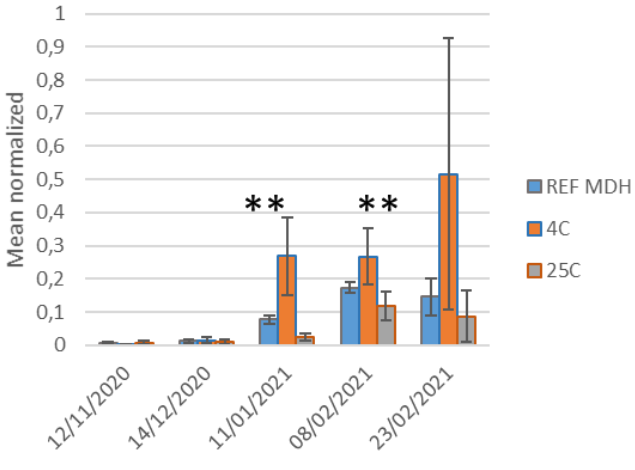

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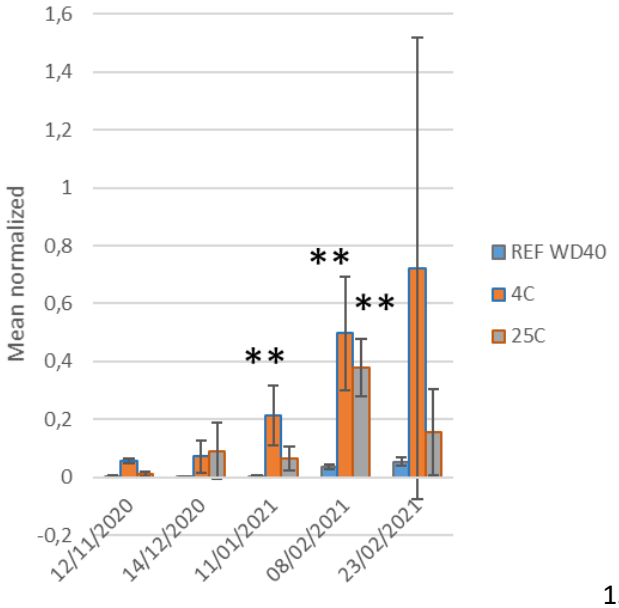

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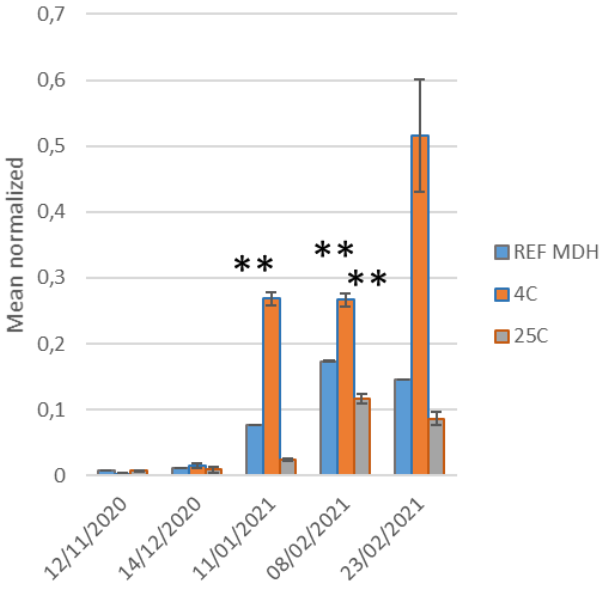
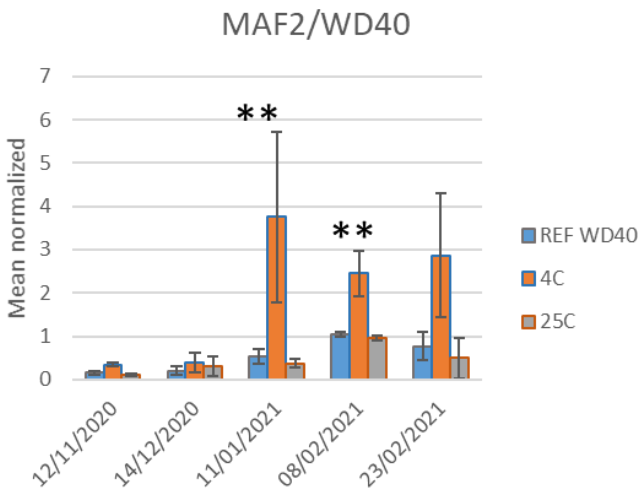
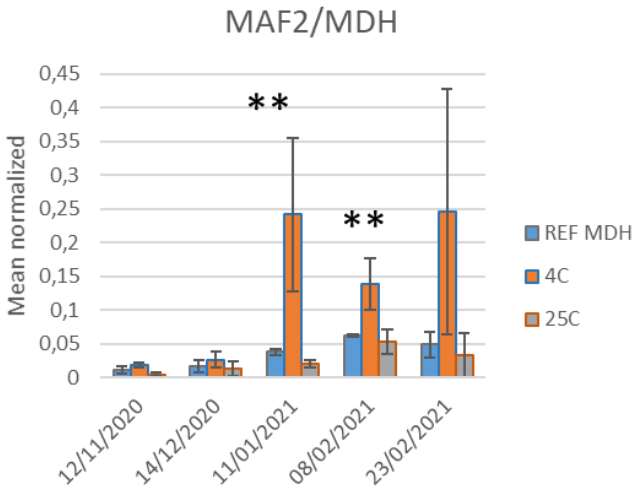


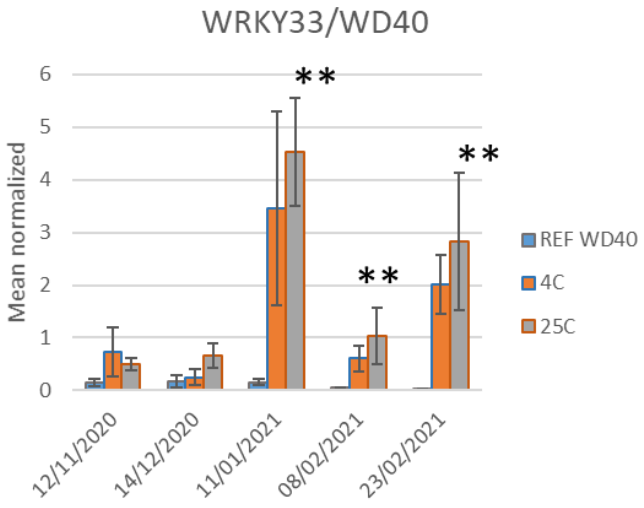
Annex II

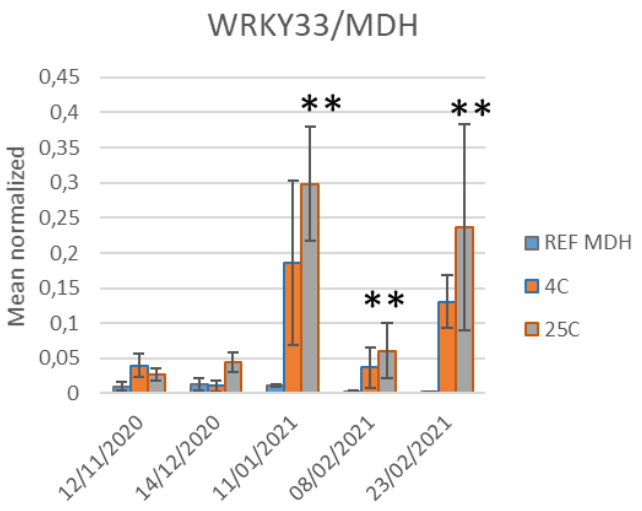
Dormancy marker genes

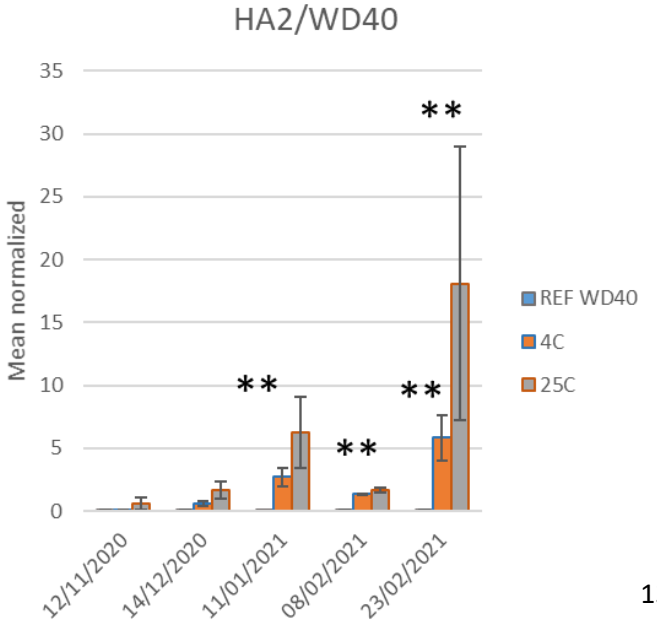


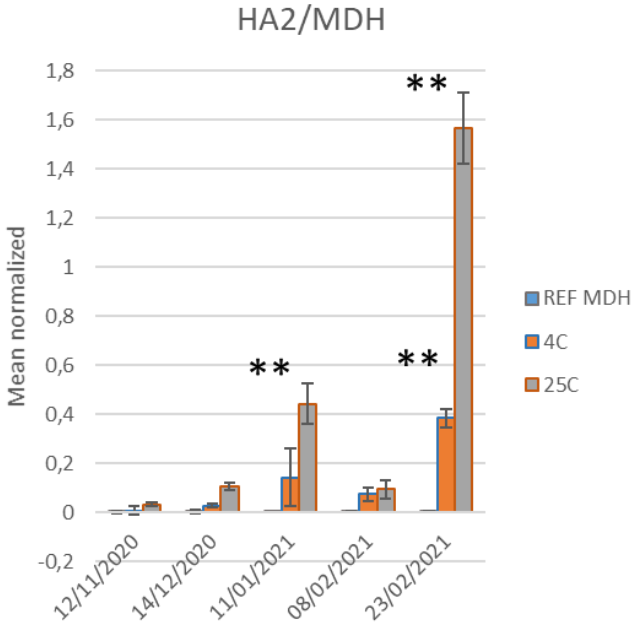


Candidate genes



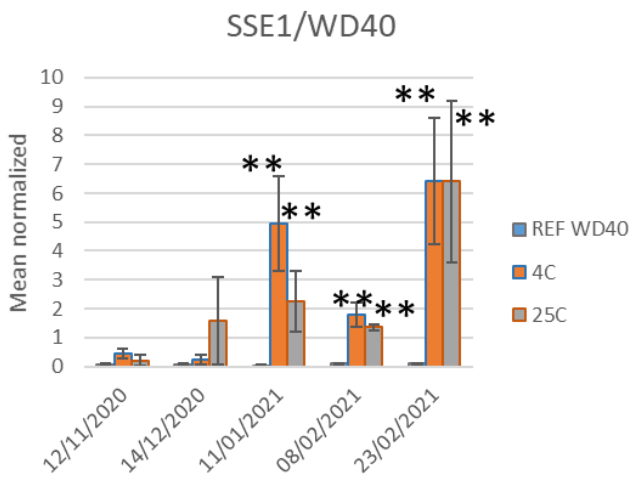


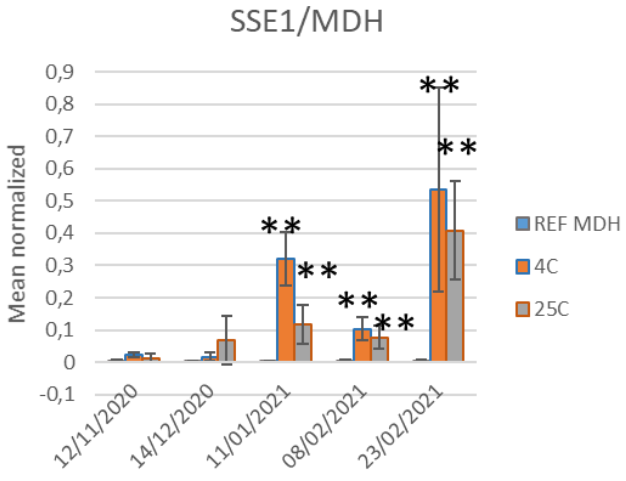


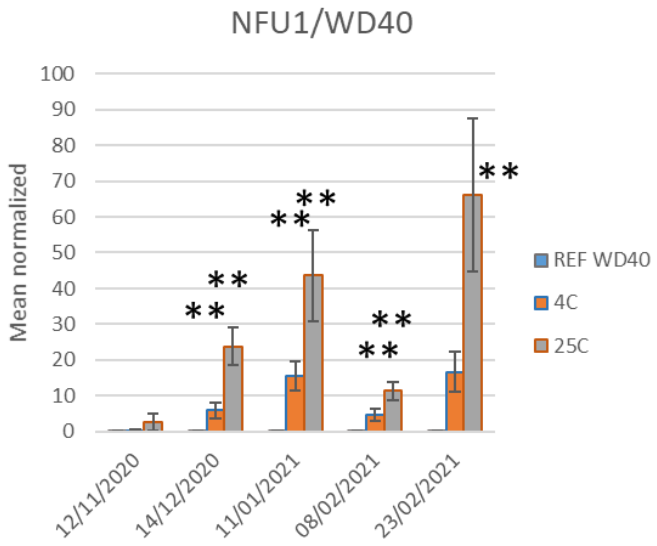


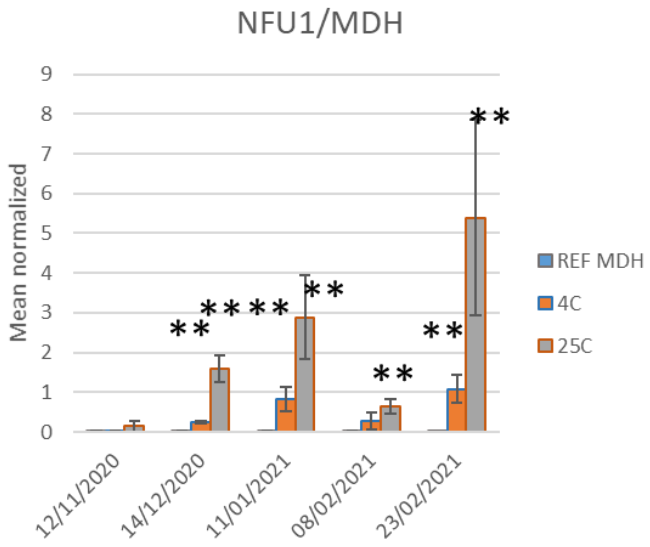
Annex II

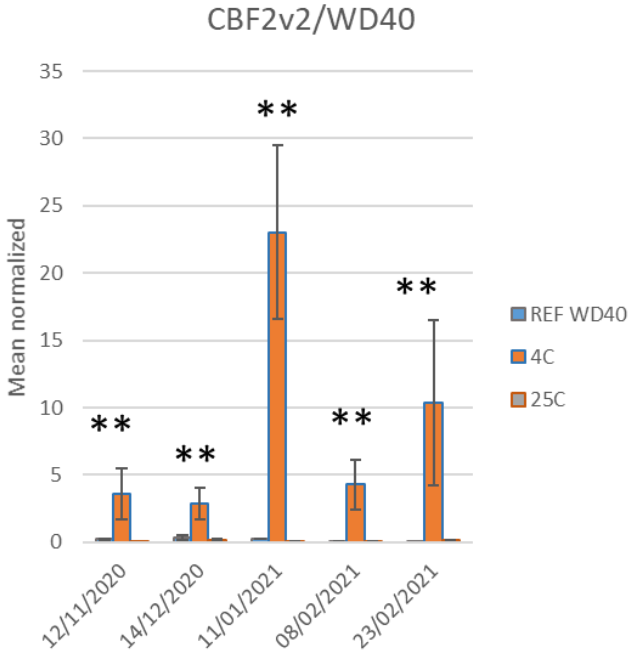
Candidate genes

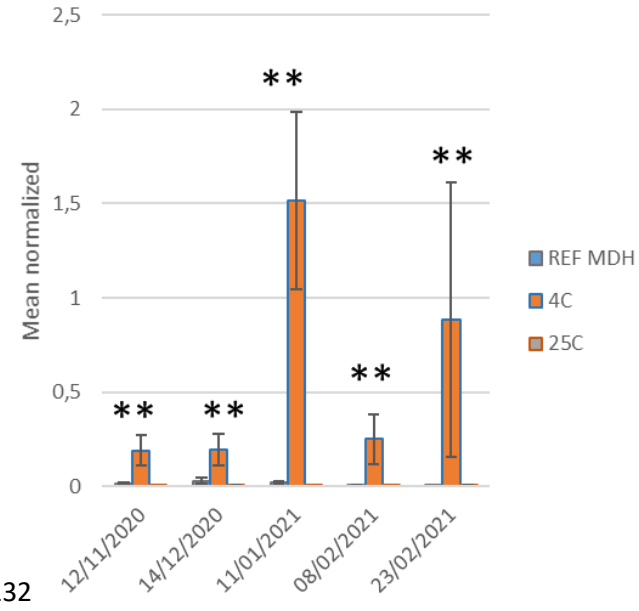






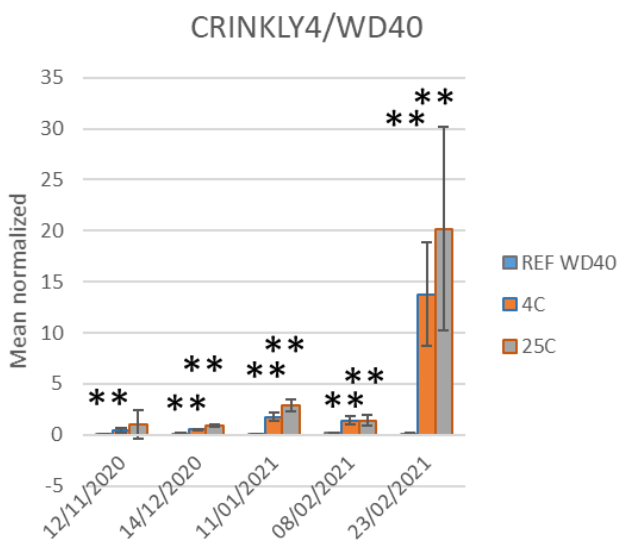


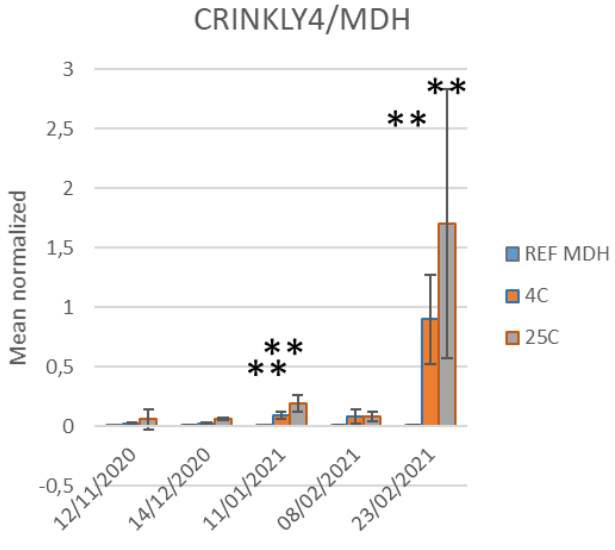


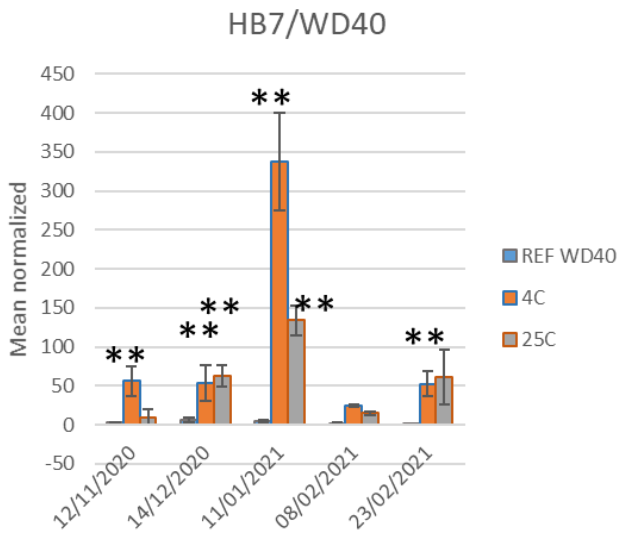


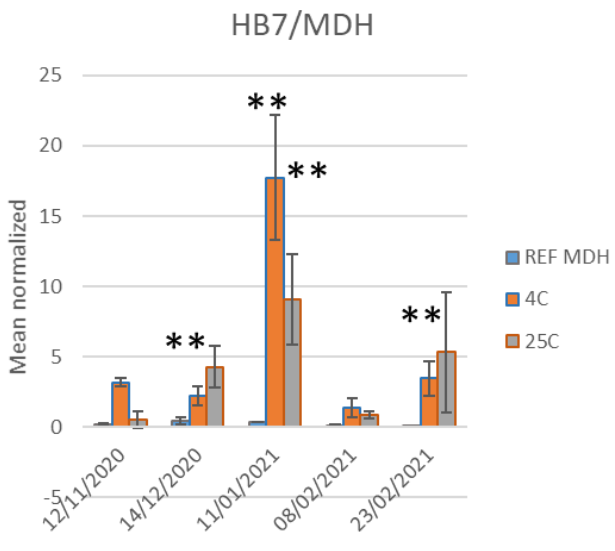
Annex II

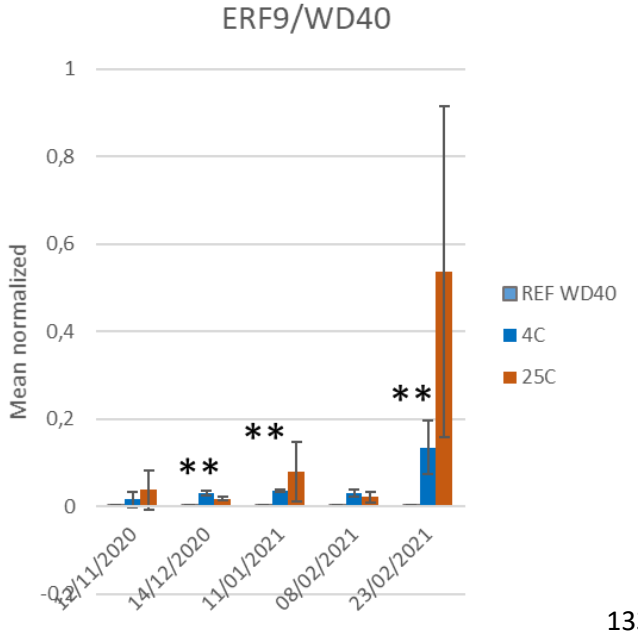
Candidate genes

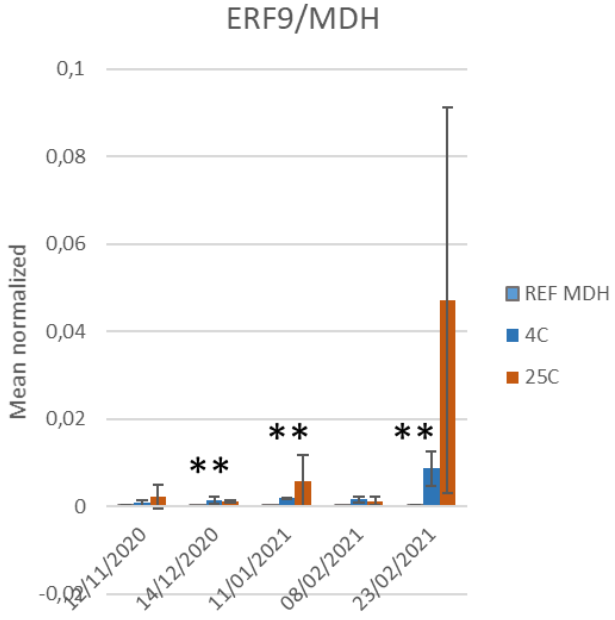






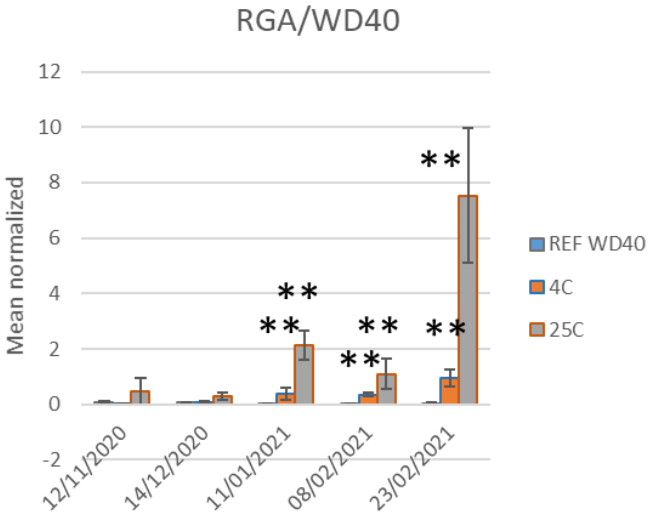


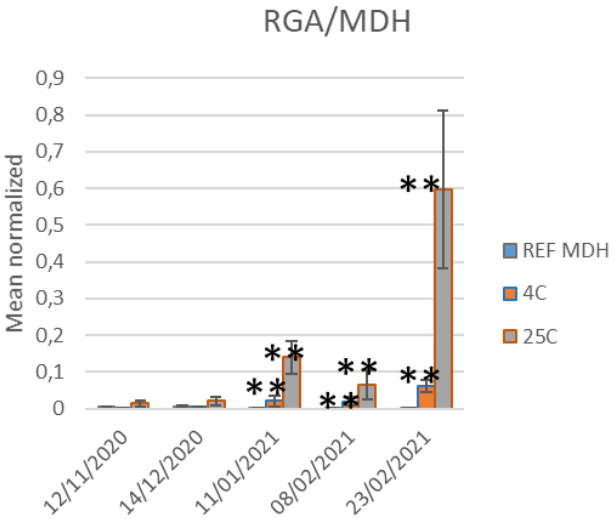


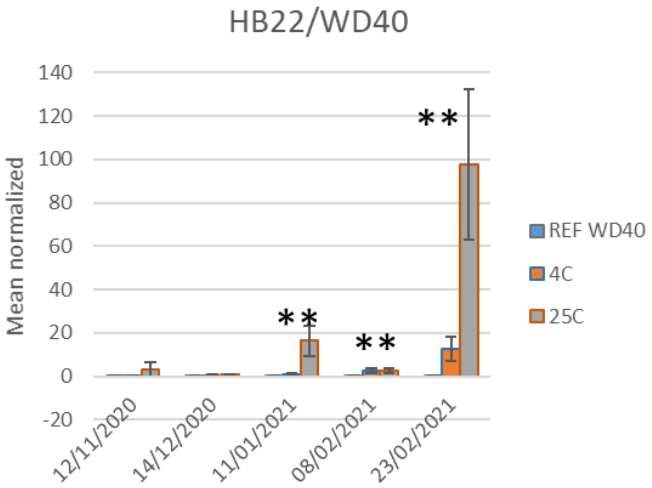


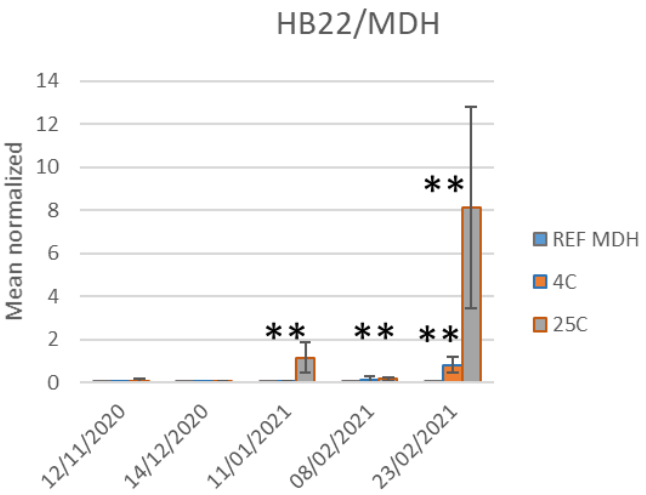
Annex II

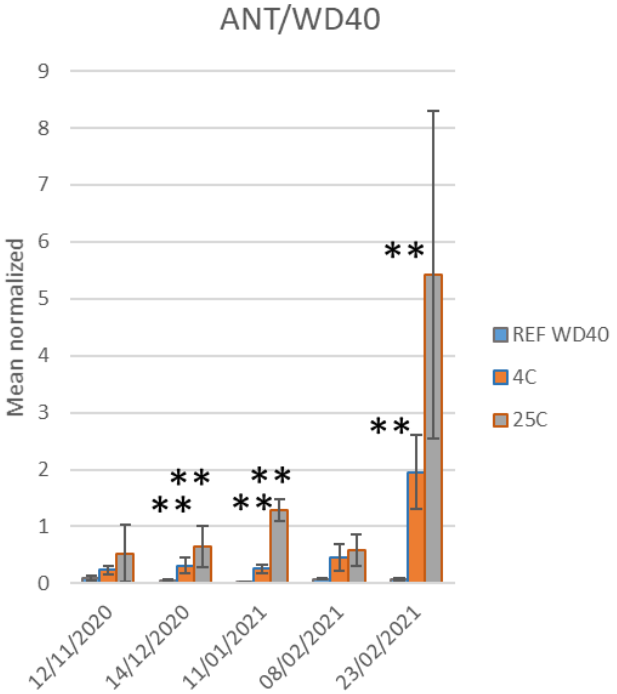
Candidate genes

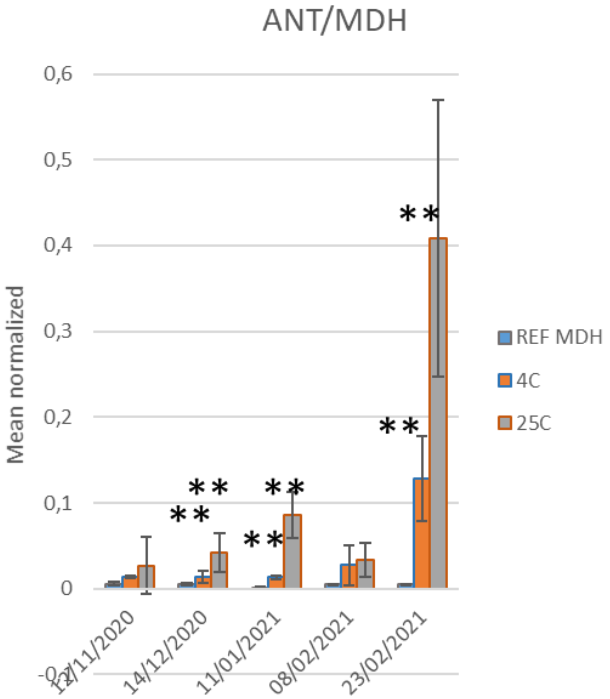






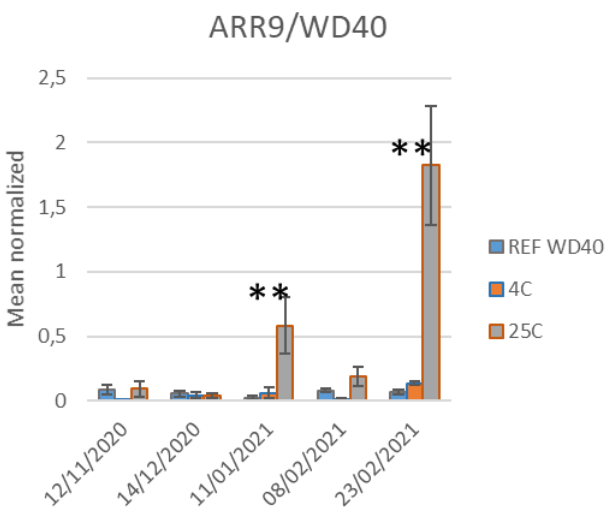


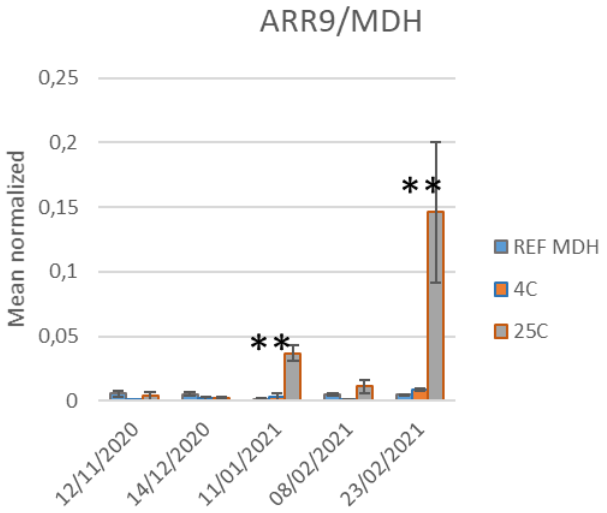


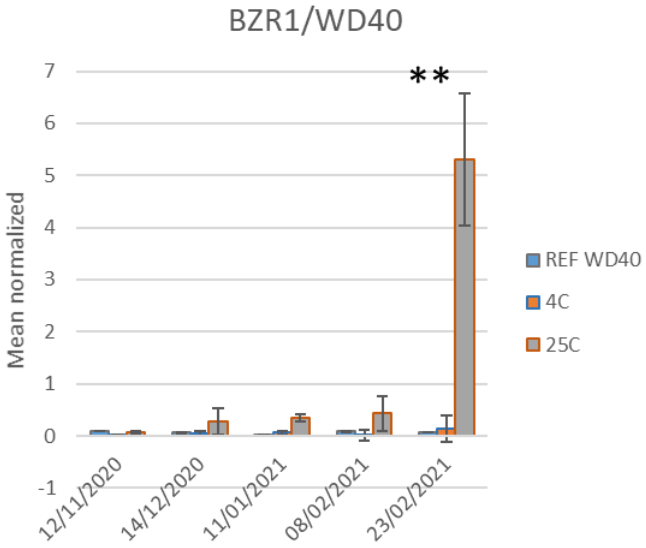


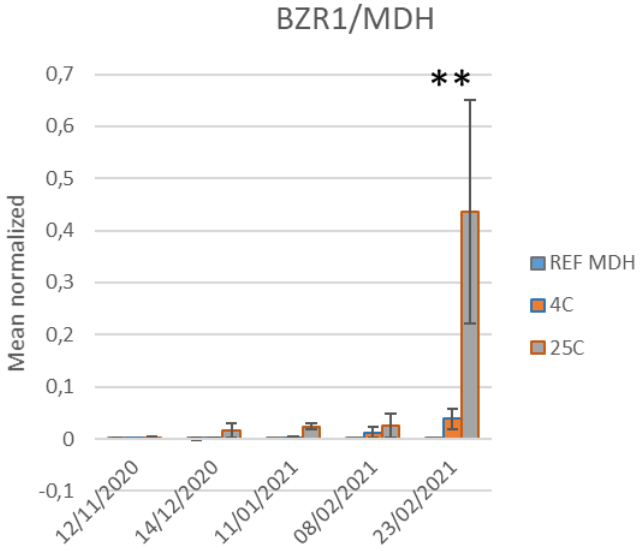
Annex II

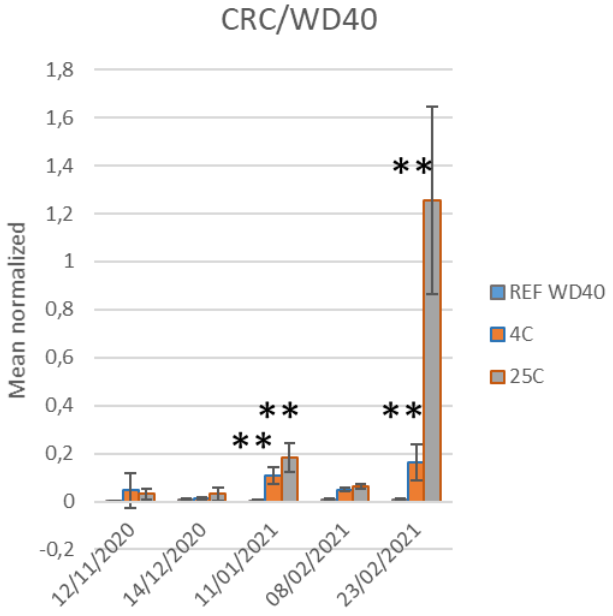
Candidate genes

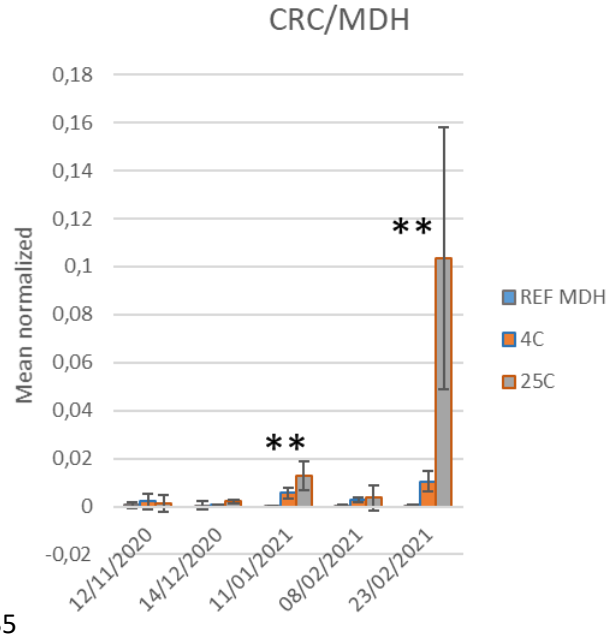












Annex II

Candidate genes

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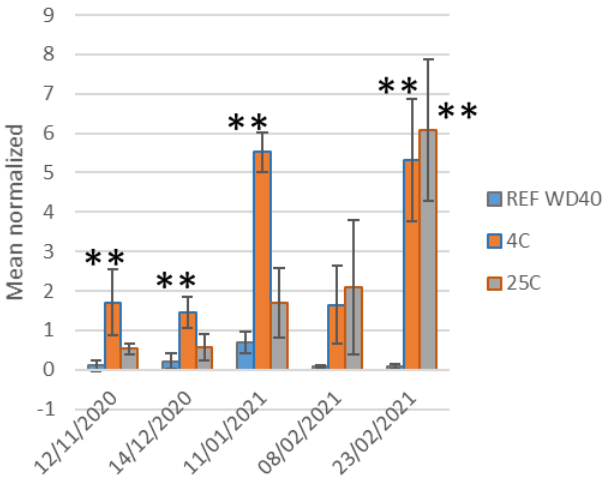

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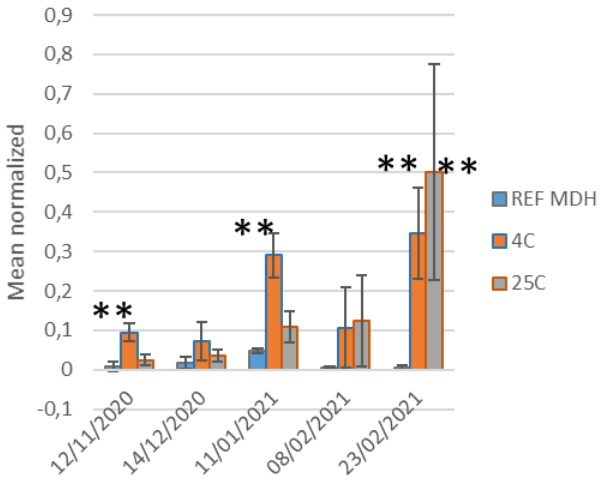

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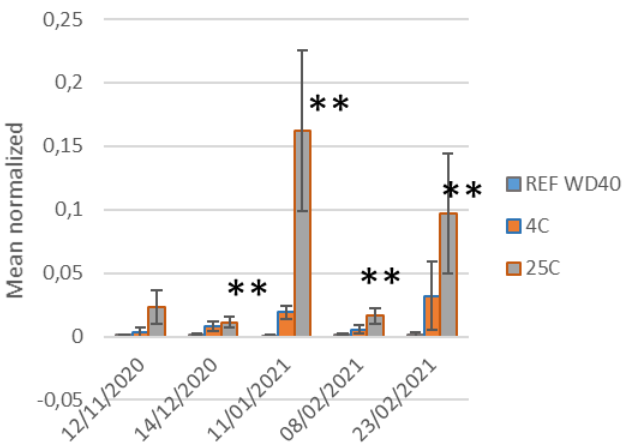

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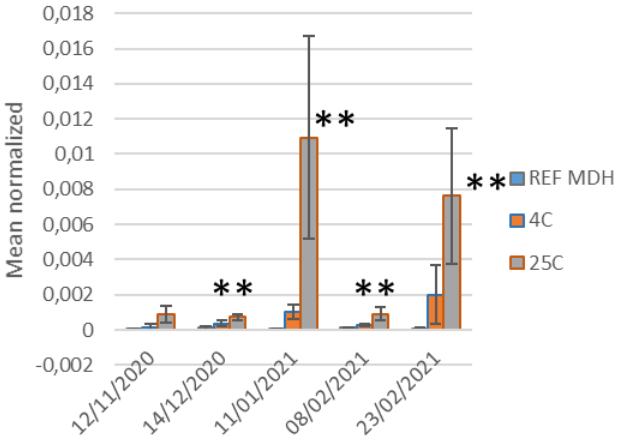

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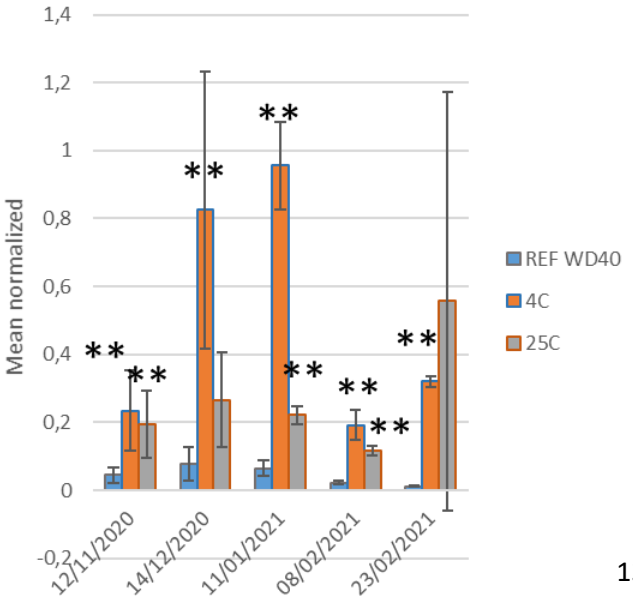

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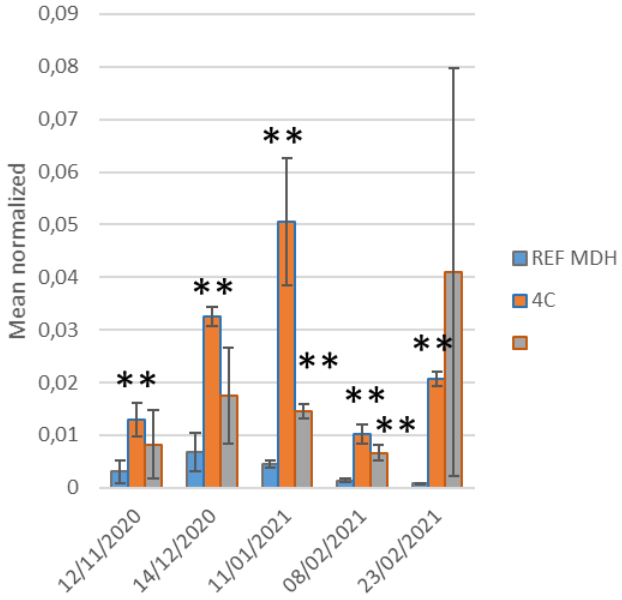
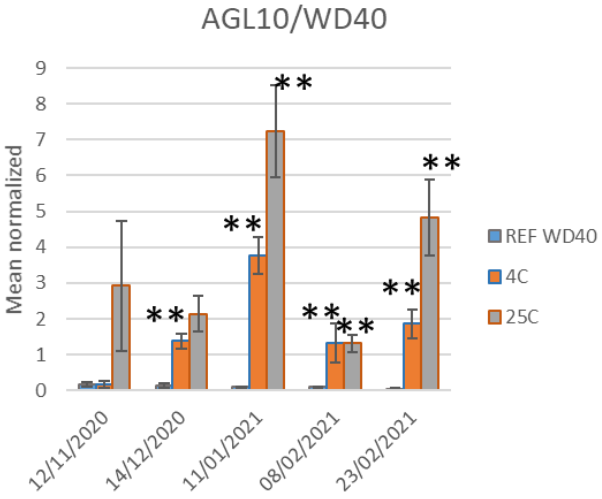
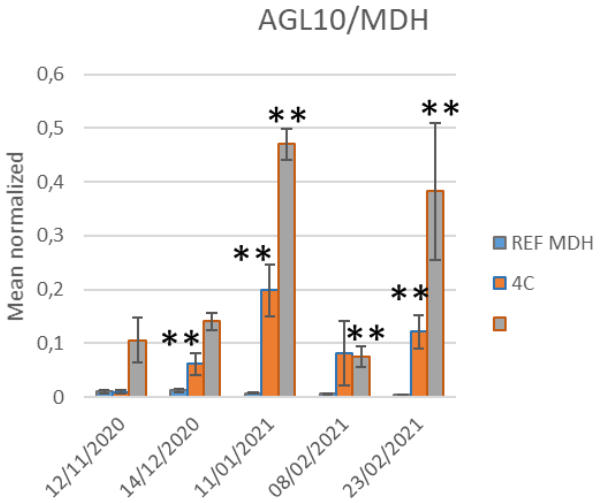
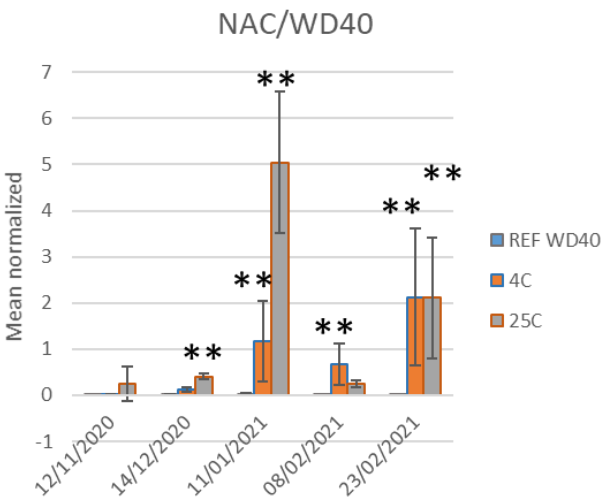


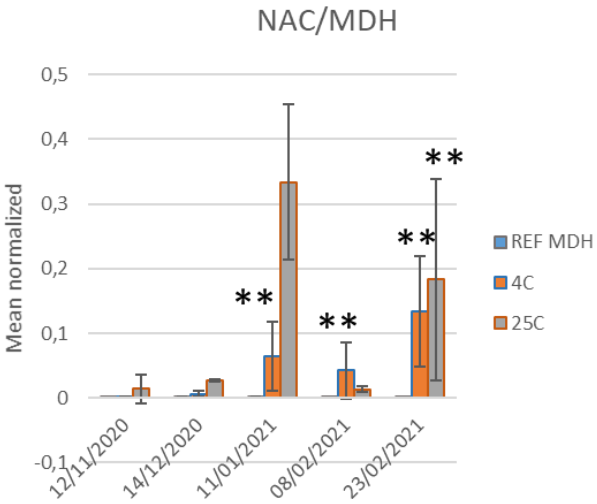
Annex II

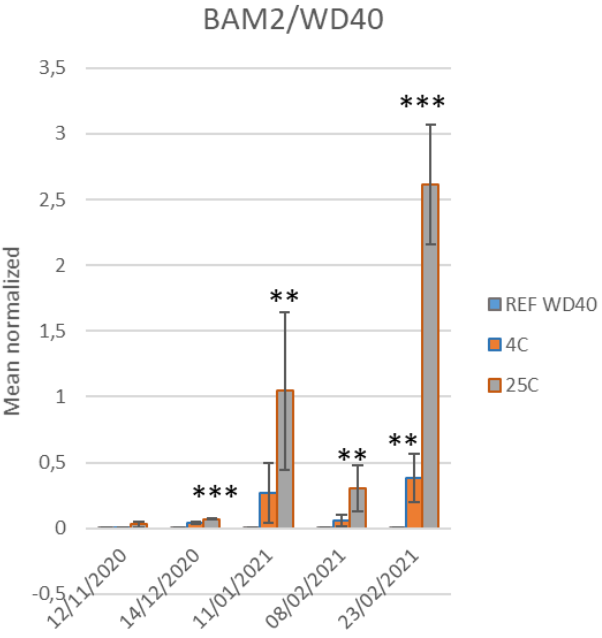
Candidate genes

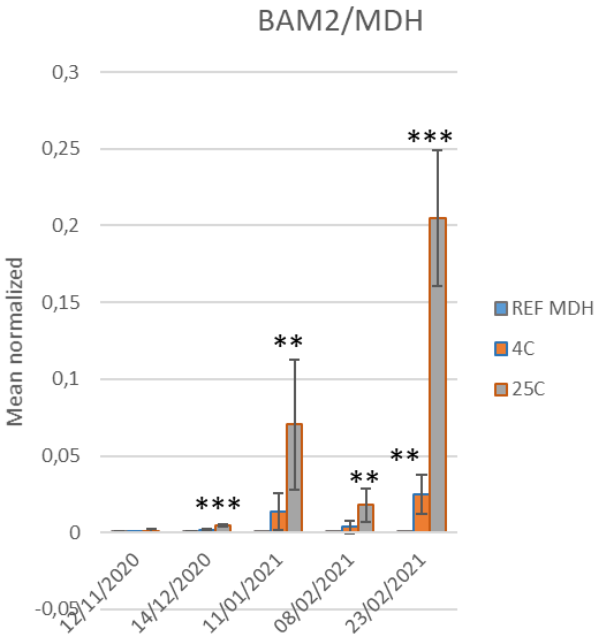






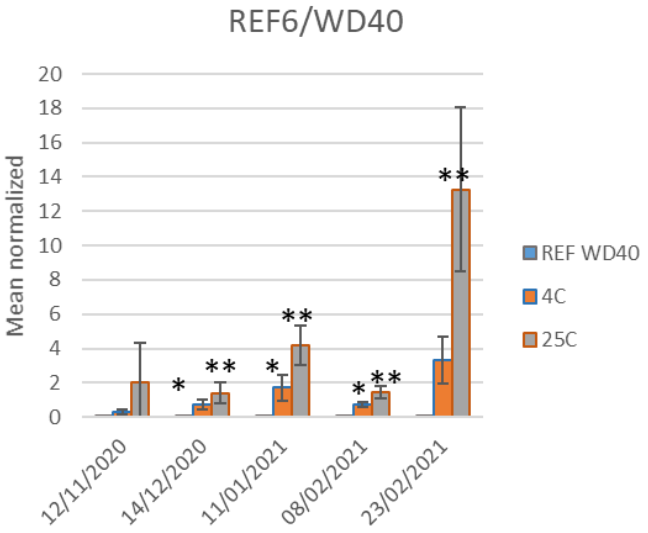


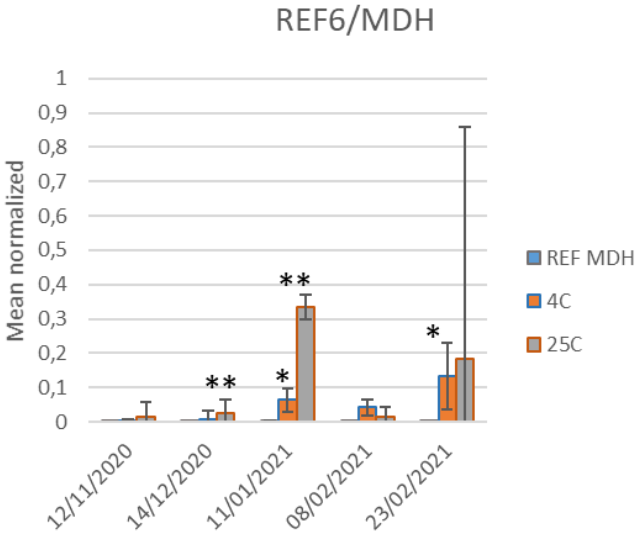


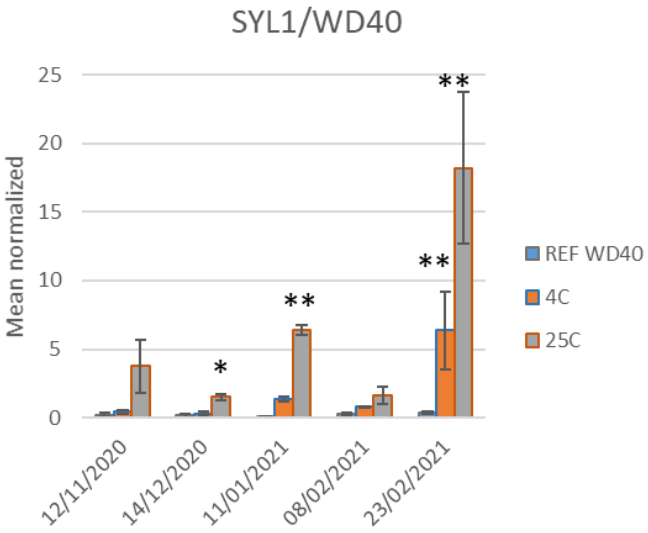


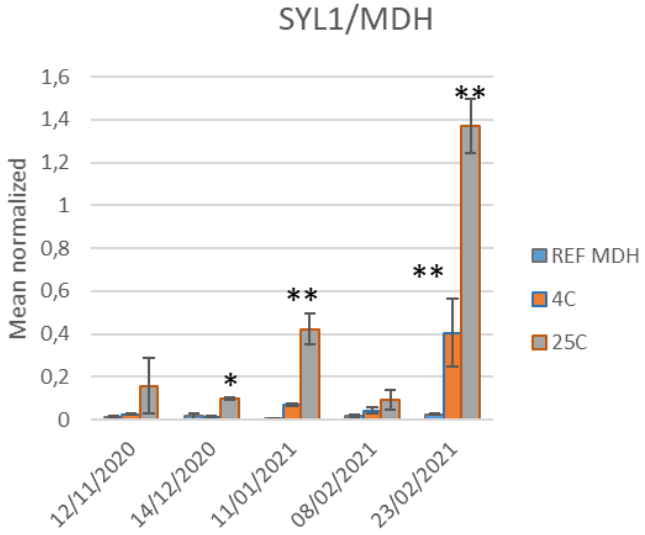
Annex II

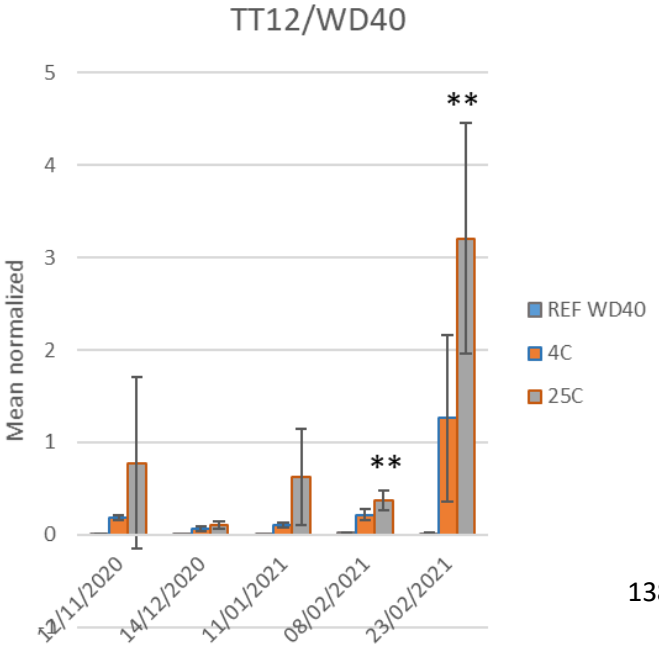
Candidate genes

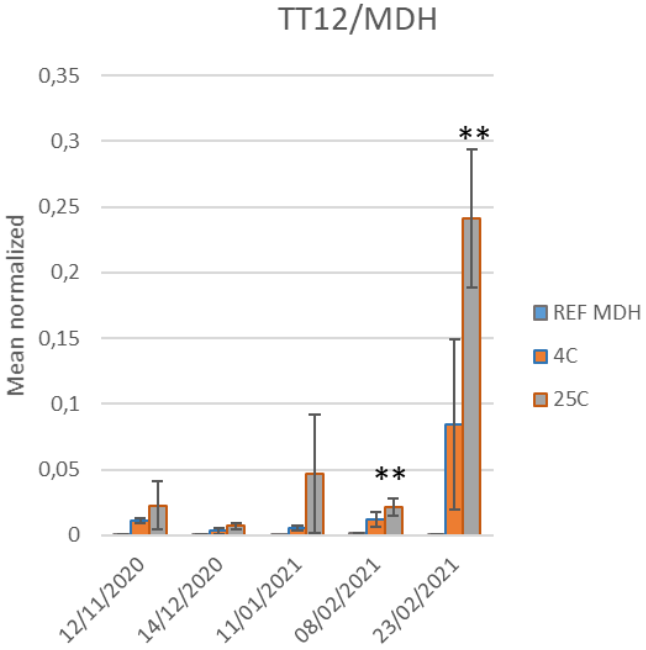












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CHAPTER 3

The Identification of Small RNAs Differentially Expressed in Apple Buds Reveals a Potential Role of the Mir159-MYB Regulatory Module during Dormancy

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ABSTRACT

Winter dormancy is an adaptative mechanism that temperate and boreal trees have developed to protect their meristems against low temperatures. In apple trees (*Malus x domestica*), cold temperatures induce bud dormancy at the end of summer/beginning of the fall. Apple buds stay dormant during winter until they are exposed to a period of cold known as a chilling requirement (CR). Once the CR is satisfied, dormancy is released and buds can resume growth (bud break) and initiate flowering in response to warm temperatures in spring.

In the annual model plant species *A. thaliana*, the thermo-regulation of flowering time is strongly controlled by the epigenetic and post-transcriptional modification of floral-regulators genes mediated by small RNAs. Small RNAs modulate temperature responses also in woody plant species, such as grapevine, poplar and pear. However, how small RNAs are involved in genetic networks of temperature-mediated to regulate dormancy and bud break in fruit tree species remains unclear.

To study the role of small RNAs in the control of apple dormancy, we performed a small RNA sequencing on bud samples during a time-course experiment. Using the method “TraPR” (Trans-kingdom, rapid, affordable Purification of RISCs), 373 different potential micro RNAs (miRNAs) were identified. A total of 233 small RNAs were annotated in public databases as known miRNAs, whereas the other 128 were annotated as unknown small RNAs. Seventeen miRNAs were differentially expressed in endodormancy and ecodormancy phases. The genes targets of these 17 miRNAs were mostly associated with functions on “Plant Development”, “Regulation of Growth” and “Response to Stimulus”. Among the genes related to “Response to Hormones”, I found several miRNA target genes related to the abscisic acid (ABA) pathway that encode MYB transcription factors. Furthermore, I studied the conservation between *A. thaliana* and apple of a regulatory miRNA/target gene module in the context of ABA homeostasis. From the results of this study, I speculated that the apple miR159 acts in the shoot apex to reduce the sensibility to ABA and prevent ABA-mediated growth inhibition at the initiation of bud break and flowering. This regulatory module could be especially important to attenuate the effect of the rise of ABA levels in growing buds caused by punctual drought and/or cold stress during the springtime.

RÉSUMÉ

La dormance hivernale est un mécanisme d'adaptation que les arbres tempérés et boréaux ont développé pour protéger leurs méristèmes contre les basses températures. Chez les pommiers (*Malus domestica*), les températures froides induisent une dormance des bourgeons à la fin de l'été/début de l'automne. Les bourgeons de pommier restent dormants pendant l'hiver jusqu'à ce qu'ils soient exposés à une période de froid connue sous le nom de besoin en froid (Chilling requirement, CR). Une fois que le CR est satisfait, la dormance est levée et les bourgeons peuvent reprendre leur croissance (débourrement) et initier la floraison en réponse aux températures chaudes du printemps.

Chez l'espèce végétale modèle annuelle *A. thaliana*, la thermorégulation de la période de floraison est fortement contrôlée par la modification épigénétique et post-transcriptionnelle des gènes régulateurs floraux et est médiée par de petits ARN. Les petits ARN modulent également les réponses à la température chez les espèces végétales ligneuses, telles que la vigne, le peuplier et le poirier. Cependant, la façon dont les petits ARN sont impliqués dans les réseaux génétiques en réponse à la température pour réguler la dormance et le débourrement des espèces d'arbres fruitiers reste incertaine.

Pour étudier le rôle des petits ARN sur le contrôle de la dormance des pommiers, nous avons effectué un séquençage de petit ARN sur des échantillons de bourgeons au cours d'une séquence temporelle. En utilisant la méthode « TraPR » (Trans-kingdom, rapid, abordable Purification of RISCs), 373 microARN potentiels et différents (miARN) ont été identifiés. Un total de 233 petits ARN ont été annotés dans les bases de données publiques en tant que miARN connus, tandis que les 128 autres ont été annotés en tant que petits ARN inconnus. Dix-sept miARN étaient exprimés de manière différentielle en phases d'endodormance et d'écodormance. Les gènes cibles de ces 17 miARN étaient principalement associés à des fonctions sur le « Développement des plantes », la « Régulation de la croissance » et la « Réponse au stimulus ». Parmi les gènes liés à la « réponse aux hormones », j'ai trouvé plusieurs gènes cibles de miARN liés à la voie de l'acide abscissique (ABA) qui codent pour les facteurs de transcription MYB. De plus, j'ai étudié la conservation entre *A. thaliana* et le pommier d'un module régulateur miARN/gène cible dans le cadre de l'homéostasie

de l'ABA. À partir des résultats de cette étude, j'ai émis l'hypothèse que le pommier miR159 agit dans l'apex de la pousse pour réduire la sensibilité à l'ABA et empêcher l'inhibition de la croissance médiée par l'ABA au début du débourrement et de la floraison. Ce module régulateur pourrait être particulièrement important pour atténuer l'effet de l'augmentation des niveaux d'ABA dans les bourgeons en croissance causée par un stress ponctuel, que ce soit une sécheresse ponctuelle et/ou un froid passager au printemps.

The Identification of Small RNAs Differentially Expressed in Apple Buds Reveals a Potential Role of the Mir159-MYB Regulatory Module during Dormancy

Julio Garighan [1,†], Etienne Dvorak [1,†], Joan Estevan [1], Karine Loridon [1], Bruno Huettel [2], Gautier Sarah [1], Isabelle Farrera [1], Julie Leclercq [1,3], Priscila Grynberg [4], Roberto Coiti Togawa [4], Marcos Mota do Carmo Costa [4], Evelyne Costes [1] and Fernando Andrés [1,*]

[1] UMR AGAP Institute, Univ Montpellier, CIRAD, INRAE, Institut Agro, F-34398 Montpellier, France; julio.garighan@inrae.fr (J.G.); etienne.dvorak@inrae.fr (E.D.); joan.estevan@inrae.fr (J.E.); karine.loridon@inrae.fr (K.L.); gautier.sarah@inrae.fr (G.S.); isabelle.farrera@supagro.fr (I.F.); julie.leclercq@cirad.fr (J.L.); evelyne.costes@inrae.fr (E.C.)
[2] Genome Centre, Max Planck Institute for Plant Breeding Research, D-50829 Cologne, Germany; huettel@mpipz.mpg.de
[3] CIRAD, UMR AGAP Institute, F-34398 Montpellier, France
[4] Bioinformatica Laboratory, Embrapa Recursos Genéticos e Biotecnologia—Cenargen, Brasilia 02372, Brazil; priscila.grynberg@embrapa.br (P.G.); roberto.togawa@embrapa.br (R.C.T.); marcos.costa@embrapa.br (M.M.d.C.C.)
* Correspondence: fernando.andres-lalaguna@inrae.fr

† Equal contribution.

Abstract: Winter dormancy is an adaptative mechanism that temperate and boreal trees have developed to protect their meristems against low temperatures. In apple trees (*Malus domestica*), cold temperatures induce bud dormancy at the end of summer/beginning of the fall. Apple buds stay dormant during winter until they are exposed to a period of cold, after which they can resume growth (bud break) and initiate flowering in response to warmer temperatures in spring. It is well-known that small RNAs modulate temperature responses in many plant species, but however, how small RNAs are involved in genetic networks of temperature-mediated dormancy control in fruit tree species remains unclear. Here, we have made use of a recently developed ARGONAUTE (AGO)-purification technique to isolate small RNAs from apple buds. A small RNA-seq experiment resulted in the identification of 17 micro RNAs (miRNAs) that change their pattern of expression in apple buds during dormancy. Furthermore, the functional analysis of their predicted target genes suggests a main role of the 17 miRNAs in phenylpropanoid biosynthesis, gene regulation, plant development and growth, and response to stimulus. Finally, we studied the conservation of the *A. thaliana* regulatory miR159-MYB module in apple in the context of the plant hormone abscisic acid homeostasis.

Keywords: dormancy; apple tree; small RNAs; miR159

1. Introduction

Temperate trees spread over geographical regions presenting wide seasonal environmental fluctuations all over the year. In order to optimize their reproductive success, they adjust their growth and flowering cycles to these recurrent conditions. This is possible thanks to environment sensing mechanisms and signaling pathways that reprogram their meristems in response to changes of photoperiod and temperature. In winter, short photoperiods and low temperatures induce growth cessation, the formation of winter buds to protect the meristematic tissues, and a phase of meristem growth inhibition named endodormancy (or winter dormancy) [1]. Endodormant buds regain their competence to grow after exposure to a certain period of low temperatures that is known as chilling requirement (CR). Once CR is fulfilled, the shoot apical meristem (SAM) undergoes an ecodormant phase. During ecodormancy, warm temperatures typical of springtime lead to growth resumption, bud break and flowering [2,3].

The Rosaceae is the third most economically important plant family in temperate regions and responsible for the major part of the total worldwide-consumed fruits [4]. Iconic Rosaceous species such as apple (*Malus domestica*), peach (*Prunus persica*) and sweet cherry (*Prunus avium*) are characterized by high CRs. This feature limits the range of latitudes at which they can be productively grown and makes them highly susceptible to global warming. However, despite the importance of a well-adjusted dormancy cycle for flowering timing and fruit production, the understanding on how this process is controlled in Rosaceous species and other significant crops is still in its infancy. A group of genes encoding MADS transcription factors (TF) are believed to be major regulators of the dormancy cycle in many Rosaceous fruit trees [3,5–7]. Some of these MADS TF are named as *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*) because of their genetic association with the non-dormant phenotype of the *evg* mutant of peach [6]. *DAM* genes belong to the MIKC^{C} type of MADS TF genes and are similar to *SHORT VEGETATIVE PHASE* (*SVP*) from *A. thaliana* [8]. They associate in transcriptional complexes that act in an intricate gene regulatory network (GRN) that control the transition between dormancy phases [3,5,9]. This GRN seems to be

regulated, at least in part, by the hormonal and environment-mediated transcriptional modulation of its key components [5,7,10]. For example, the plant hormone abscisic acid (ABA) is a positive regulator of dormancy induction in several tree species [11–13]. Although some recent studies have shed light on mechanisms of transcriptional regulation of bud dormancy in trees, how it is controlled at the post-transcriptional level remains unclear.

Most of the plant small RNAs consist in 21 to 24-nucleotide RNA molecules produced by DICER-LIKE (DCL) proteins [14]. Small RNAs are loaded onto ARGONAUTE (AGO)-like proteins to induce mRNA cleavage, translational repression, chromatin compaction or DNA elimination [15–17]. They are widely involved in the regulation of plant development, reproduction, responses to the environment, biotic and abiotic stress and genome reprogramming [18,19]. The micro RNAs (miRNAs) are a class of small RNAs that in plants mediate post-transcriptional gene silencing (PTGS) by transcript cleavage or translational repression [18]. In *A. thaliana*, the miR156 and miR172 are key regulators of flowering through the post-transcriptional regulation of *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) and *APETALA2*-like (*AP2*-like) genes, respectively [20–22]. Interestingly, miR156 and miR172 were found to be also involved in seed dormancy in lettuce (*Lactuca sativa*) and *A. thaliana* [23]. However, reports describing the role of small RNAs in bud dormancy are scarce. Only a few recent studies using genome-wide small RNA sequencing (small RNA-seq) identified potential miRNAs that regulate dormancy in trees [24–27]. In tree peony, several miRNAs were found to be differentially accumulated in buds between endodormancy and ecodormancy after a period of chilling. Among the identified miRNAs, five of them (miR156k, miR159a, miR167a, miR169a and miR172a) showed an inverse expression pattern as compared to their target genes [27], suggesting their role during endodormancy to ecodormancy transition in tree peony. Another recent study reported the existence of miRNA-mediated regulating the photoperiod-dependent dormancy induction in Vitis species [28]. Most of the studies concerning Rosaceous species have been done on pear and peach [24,26,29,30]. More than hundred miRNAs were expressed in buds during dormancy of Japanese pear (*Pyrus pyrifolia* ‘Kosui’) [26]. In white pear (*Pyrus pyrifolia*), it was found that miRNA6390 targeted several *DAM* genes as a part of a potential mechanism of endodormancy release [24]. A *Prunus*-specific miRNA (miR6285) was also found to be differentially

expressed between endodormancy and ecodormancy in peach. MiR6285 targets an asparagine-rich protein (NRP) that is involved in the regulation of ABA signaling in *A. thaliana* [29].

Here, we have made use of a recently developed AGO-purification technique [31] to isolate small RNAs from apple buds. Combining small RNA-seq and public RNA-seq data allowed us to identify miRNA and their target genes potentially involved in the control of dormancy cycle of fruit trees. Furthermore, we studied the conservation between *A. thaliana* and apple of a regulatory miRNA/target gene module in the context of the ABA homeostasis.

2. Results

2.1. Different Types of Known and Unknown Small RNAs Are Expressed in Apple Buds during the Dormancy Cycle

In order to identify small RNAs expressed during dormancy cycle in apple, bud samples were collected at different dates covering the transition between endodormancy to ecodormancy. Four dates were selected according to the physiological stage of the buds (Table 1 and Figures 1A,B). The physiological stage of the buds was defined using a forcing test (Tabuenca test [32,33]) that allowed us to distinguish between endodormant and ecodormant buds (Figure 1B). Based on the Tabuenca test that we periodically performed between November 2018 and March 2019, we determined that the transition from endodormancy to ecodormancy occurred around 04/02/2019 (Stage 2, Table 1). Before this date, buds remained fully endodormant (Stage 1), whereas after this date, buds became ecodormant (Stages 3 and 4), i.e., competent to resume growth upon exposure to warm T° (Figure 1A and Table 1).

Table 3-1 Samples used for the small RNA and sequencing results

Sample	Date	Avg T (°C)	CH (h)	Phase	Library	Reads
					A	22,353,097
Stage 1	7 January2019	6.00	665	Early endo	B	23,372,379
					C	22,980,048
					D	20,623,140
Stage 2	4 February 2019	5.84	1098	Late endo	E	23,353,672
					F	18,094,050
					G	23,560,798
Stage 3	19 February 2019	7.98	1280	Early eco	H	16,446,157
					I	20,745,045
					J	20,396,876
Stage 4	04 March 2019	11.28	1395	Late eco	K	22,812,563
					L	21,813,675

Sample: name of the sample, Date: date of sampling, Avg T: average of the day-temperature at the sampling date, CH: chilling hours at the sampling date, Phase: physiological stage of the trees based on a forcing test (endo: endodormant, eco: ecodormant), Library: name of the library for sequencing, Reads: number of reads obtained per library.

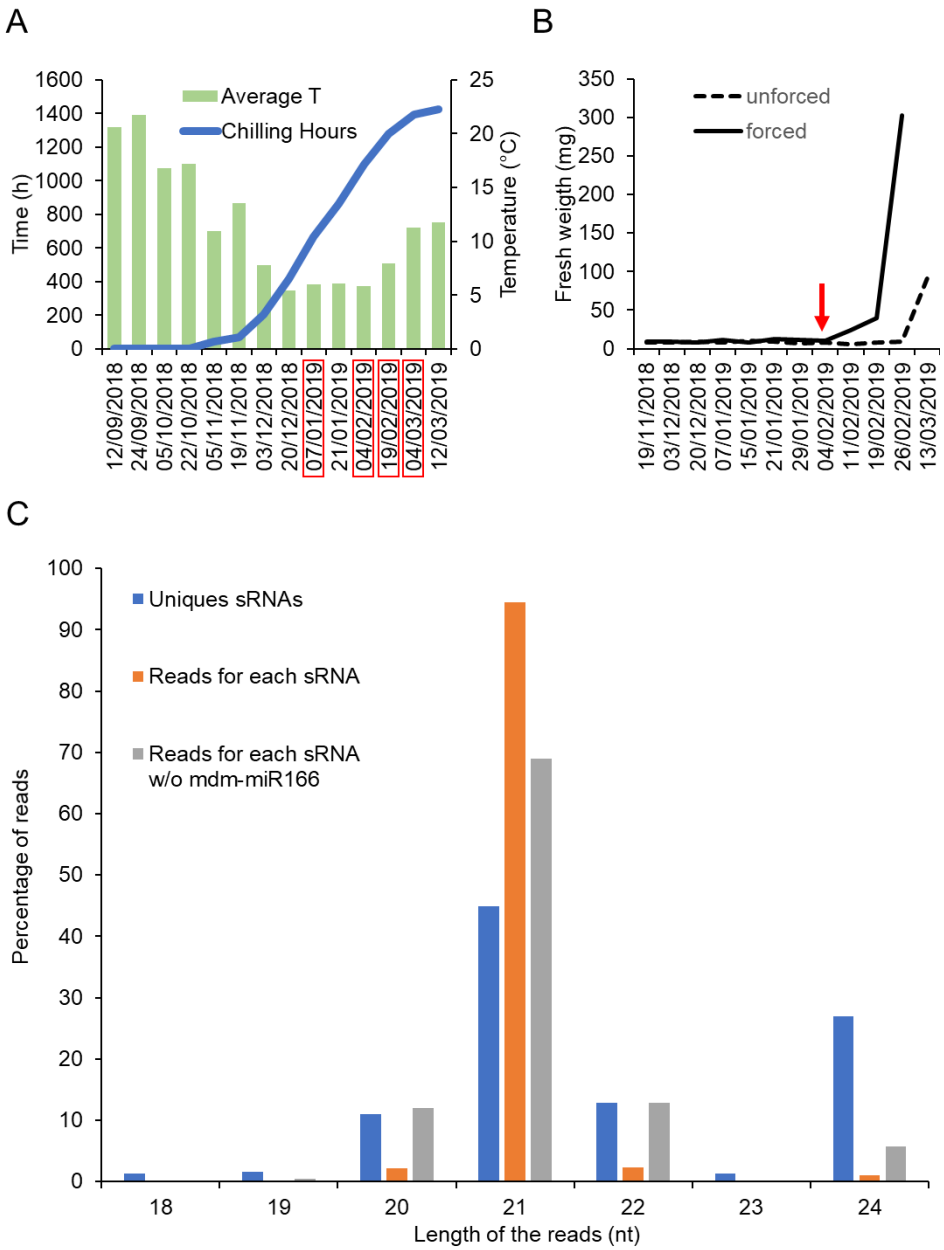

Figure 3-1 Identification of small RNAs expressed during dormancy of apple buds. (A) Average temperature and chilling hours accumulation during the experiment done between 2018 and 2019. (B) Result of the Tabuenca test showing the around which endodormancy is released (red arrow) (C) Percentage of reads corresponding to the number of distinct types of small RNAs (Unique sRNAs), to the nucleotide lengths distribution (Reads for each sRNA) with or without mdm-miR166 (Reads for each sRNA w/o mdm-mR166).

To isolate small RNAs from these samples, we have made use of a recently developed method called “TraPR”, which purification protocol is based on AGO’s ability to associate with small RNA. The sequencing results after small RNA isolation using this method are shown in Table 1. A total of 372 different small RNAs were detected across samples, among which, 361 corresponded to biologically relevant small RNAs

(i.e., length between 20 to 24 nucleotides) (Table S1). The size distribution shown in Figure 1C indicates a predominant abundance of 21-nucleotides small RNAs. Notably, around one third (28%) of the detected unique small RNAs corresponded to 24-nucleotides small RNAs, which could mediate transcriptional silencing of transposons and pericentromeric repeats via the RdDM pathway [18]. A total of 233 small RNAs were annotated in public databases as known miRNAs, whereas the other 128 were annotated as unknown small RNAs (Table S1). Particularly abundant were the miRNAs belonging to the miR166, miR159, miR482, miR1511, miR319 and miR171 families. Remarkably, only the miR166 accounted for around 80% of the total recovered reads (Table S1).

2.2. Several miRNAs Are Differentially Expressed between Endodormancy and Ecodormancy in Apple

We tested for small RNA differential expression using two R packages (DESeq2 and Edge2). An analysis performed with DESeq2 resulted in the detection of 26 differentially expressed small RNAs (DE-small RNAs), while EdgeR found 33 DE-small RNAs (Supplementary Table S2; *p*-value ≤ 0,05). A group of 20 DE-small RNAs was common between the two analyses (Figure 2A). This group was considered as a high confidence list of DE-small RNAs. The high confident list contained five small RNAs (t11329296_x5768, t05229383_x4081, t08515395_x8579, t00205877_x43290 and t00969484_x10829) that were not included in any public miRNA database. Therefore, we performed a further analysis to evaluate their stem-loop structures. In silico RNA folding analyses predicted that only t08515395_x8579 and t00205877_x43290 have an optimal stem-loop structure compatible with biologically functional miRNAs [34] (Figure 2B). Therefore, the other three small RNAs (t11329296_x5768, t05229383_x4081 and t00969484_x10829) were discarded for downstream studies. Distinct patterns of expression were observed within the 17 DE-miRNAs of the high confidence list (Table 2 and Figure 2C). Four miRNAs were upregulated at Stage 4 (grey bar in Figure 2C). Two of them, mdm-miR159a and mdm-miR5225c increased their expression at the transition between endodormancy to ecodormancy. Similarly, a group of seven miRNAs (orange bar) were upregulated at Stage 3 but their expression levels dropped at Stage 4. Three miRNAs displayed reduced levels of expression at Stage 4 compared to Stages 1 and 2 (blue bar) and other three ones showed two peaks of expression at Stages 1 and 3 (green bar).

Table 3-2 Top DE-miRNAs

	EDGER				DESEQ2			
Name (ID-Accession)	**S2 vs. S1**	**S3 vs. S1**	**S4 vs. S1**	***p* Value**	**S2 vs. S1**	**S3 vs. S1**	**S4 vs. S1**	***p* Value**
ath-miR858b	−0.70	1.12	−0.16	3.45×10^{-3}	−0.56	1.17	0.01	1.04×10^{-3}
mdm-miR159aMIMAT0025898	0.72	1.94	1.64	4.37×10^{-3}	0.89	1.99	1.93	1.24×10^{-3}
mdm-miR164cMIMAT0025909	−0.90	−0.22	−1.98	3.26×10^{-3}	−0.76	−0.17	−1.77	3.71×10^{-3}
mdm-miR171bMIMAT0025939	−0.83	0.74	−0.94	2.02×10^{-3}	−0.70	0.78	-0.78	6.28×10^{-4}
mdm-miR390eMIMAT0025973	−0.51	0.08	1.51	9.33×10^{-4}	−0.44	0.16	1.70	3.99×10^{-6}
mdm-miR482bMIMAT0026022	−0.75	0.30	−1.19	1.48×10^{-2}	−0.63	0.33	−1.03	1.19×10^{-2}
mdm-miR482cMIMAT0026023	−0.93	−0.04	−1.23	2.89×10^{-2}	−0.81	−0.01	−1.07	2.47×10^{-2}
mdm-miR5225cMIMAT0026052	−0.83	1.54	1.40	5.60×10^{-6}	−0.70	1.56	1.60	4.76×10^{-8}
mdm-miR7121cMIMAT0026042	−0.31	0.94	−0.59	1.17×10^{-2}	−0.16	1.01	−0.35	4.83×10^{-3}
mdm-miR7121eMIMAT0026044	0.30	1.59	−0.72	1.37×10^{-3}	0.44	1.62	−0.52	1.02×10^{-3}
mdm-miR858MIMAT0026070	0.59	2.51	−0.26	3.81×10^{-4}	0.77	2.57	−0.05	3.12×10^{-3}
mtr-miR4414a-5p	0.59	1.88	0.29	2.22×10^{-3}	0.75	1.94	0.47	9.98×10^{-4}
peu-miR2910	−0.01	1.33	0.17	2.05×10^{-2}	0.11	1.37	0.39	6.48×10^{-3}
ptc-miR159d	−0.11	0.70	1.92	2.53×10^{-4}	0.02	0.77	2.16	1.78×10^{-7}
sit-miR05-npr	0.44	−0.99	−0.54	4.77×10^{-2}	0.56	−0.94	−0.26	2.67×10^{-2}
t00205877_x43290	0.45	0.31	−1.00	1.33×10^{-2}	0.59	0.38	−0.79	3.48×10^{-3}
t08515395_x8579	0.20	−0.01	−1.67	6.17×10^{-3}	0.31	0.03	−1.43	7.52×10^{-3}

Name (ID-accession): ID and accession according to the miRBase nomenclature. S1: Stage 1; S2: Stage 2; S3: Stage 3; S4: Stage 4. Numeric values correspond to Log2 of the Fold Change (Log2FC) between stages.

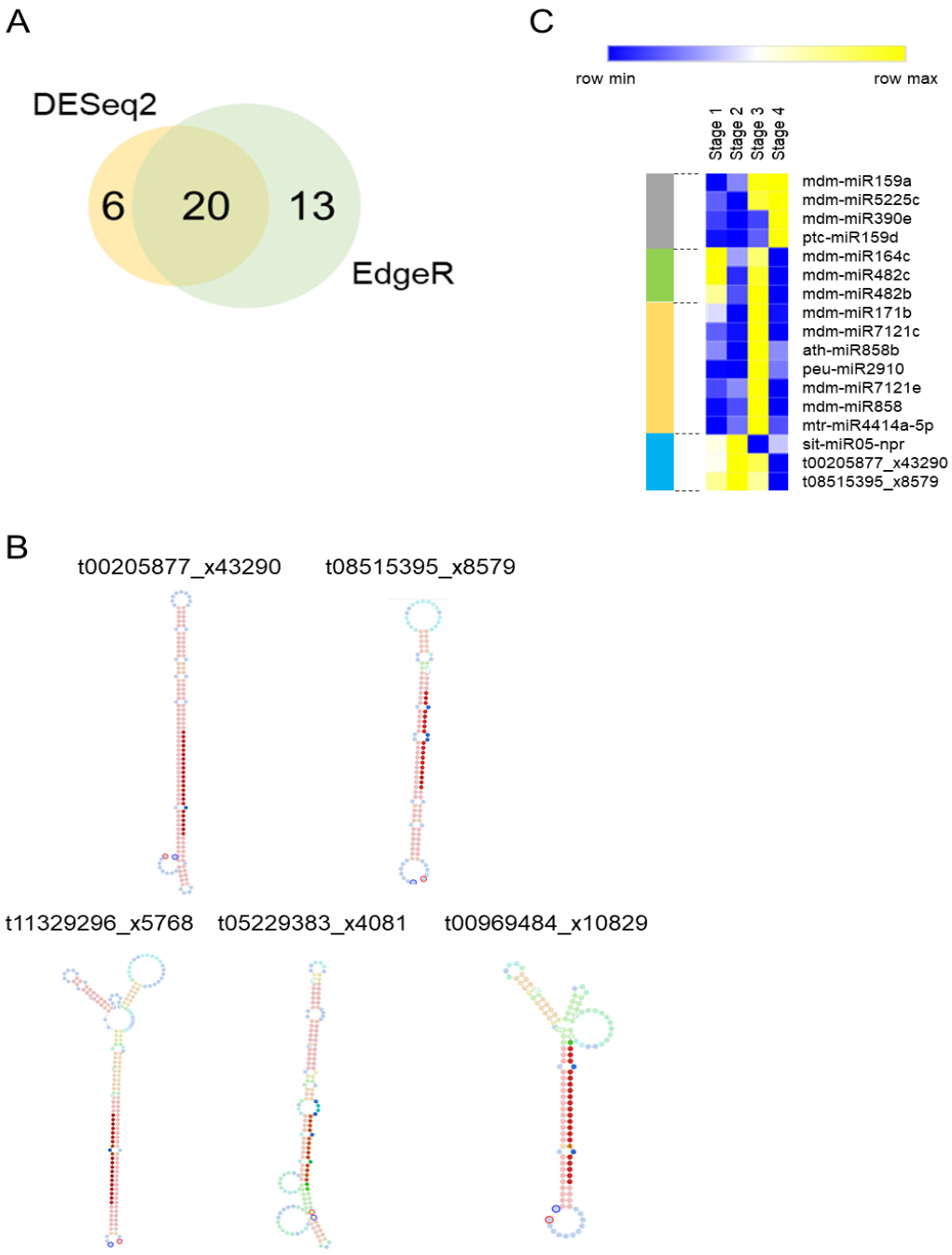

Figure 3-2 Several small RNAs are differentially expressed between endodormancy to ecodormancy in apple buds. (A) Venn Diagram comparing the results between the EdgerR and Deseq2 analyses. (B) Heat map Expression profile of the 17 DE-miRNAs. (C) Stem-loop structure prediction of unknown DE-small RNAs.

2.3. Predicted Target Genes of DE-miRNAs Are Involved in Key Regulatory Biological Processes

We made use of the psRNATarget web server for the identification of target genes of the DE-miRNAs. This analysis resulted in the identification of 420 potential miRNA target genes (Table S3). One quarter of these genes (101 genes) were common

targets among the 17 DE-miRNAs. Next, we made use of public RNA-seq data [35] to represent the expression profile of the 319 unique target genes in a heat map. Several target genes displayed changing expression patterns in apple buds collected at distinct dates during winter dormancy (Figures 3A and S1), suggesting their possible role in this biological process. To investigate the biological function of these target genes, we performed a gene ontology (GO) enrichment analysis. This analysis resulted in the classification of the 319 target genes into five main categories related to plant development, regulation of gene transcription, phenylpropanoid biosynthesis, regulation of growth and response to stimulus (Figure 3B and Table S4). Categories related to plant development included genes involved in meristem and flower development, as well as xylem formation and specification of symmetry. Remarkably, genes with roles related to response to hormones, i.e., ABA, jasmonic acid, gibberellin and auxin, were overrepresented in the category response to stimulus. Moreover, the category regulation of growth included genes related to cell development and proliferation among others. Finally, several genes encoding transcription factors were found within the category of regulation of gene expression.

We found that 18% of the genes showing a GO enrichment (57 genes) were annotated as MYB transcription factors. These MYB-encoding genes were targeted by many of the 17 DE-miRNAs, suggesting their role in a post-transcriptional network regulating bud dormancy. Corroborating this idea, several of these *MYB* genes were differentially expressed during the dormancy cycle (Figure 3C).

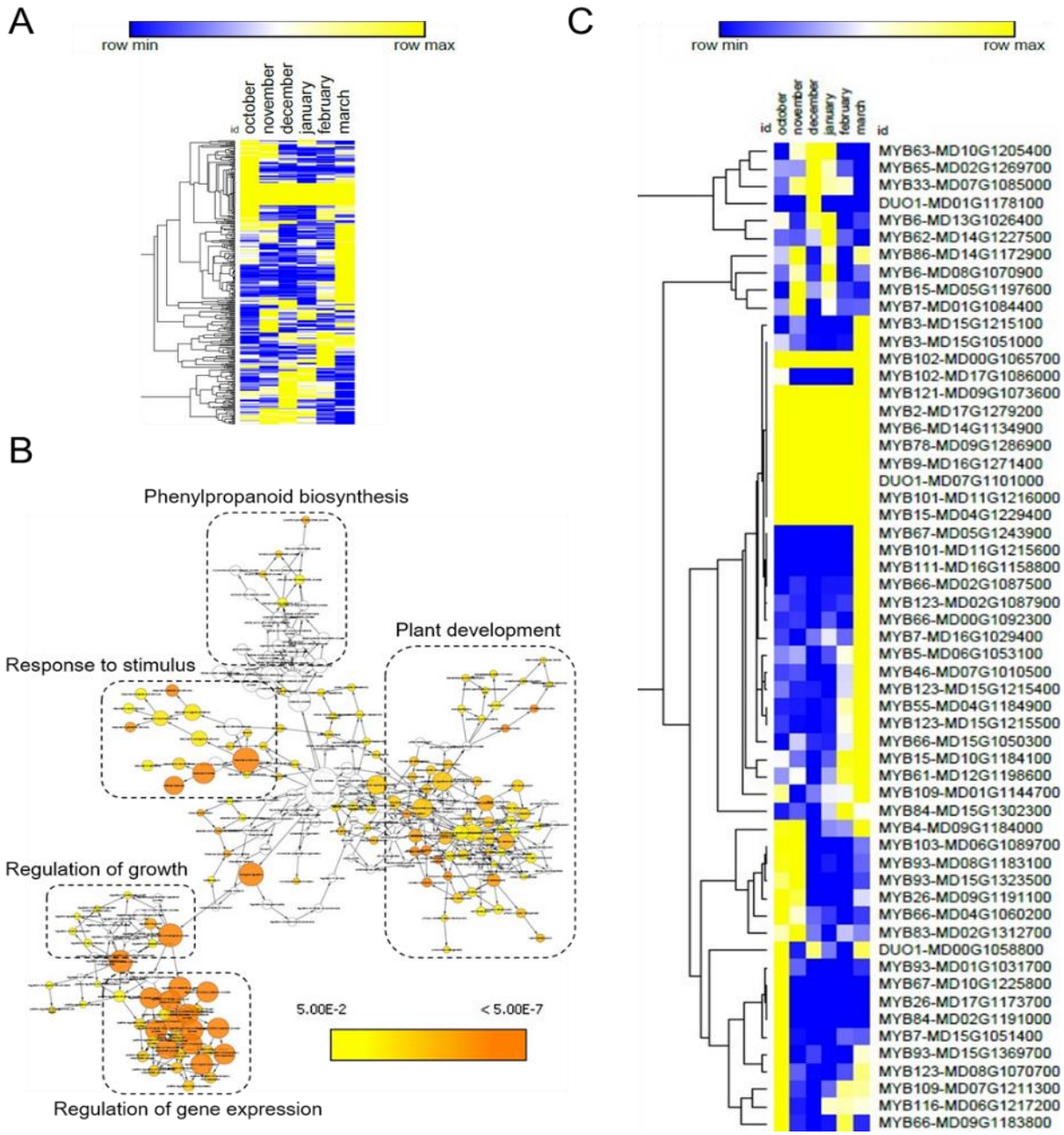

Figure 3-3 GO enrichment of DE-miRNA targets. (A) Heat map of DE-miRNA target genes patterns of expression. (B) Schematic map showing the GO enrichment of the DE-miRNA target genes. A high-resolution image is available in the Figure S1. (C) Heat map of target genes of DE-small RNA encoding MYB transcription factors. In (A) and (C), blue and yellow colours mean upregulation and downregulation, respectively.

2.4. The ABA Regulatory Module Mir159a-MYB33/MYB65 Is Conserved in Apple

Our GO analysis suggested a role of ABA in apple bud dormancy control mediated by miRNAs. ABA is a well-known hormonal regulator of dormancy in tree species [13,36,37]. Interestingly, one of the identified 17 apple DE-miRNAs is annotated as

mdm-miRNA159a, which homolog in *A. thaliana* modulates ABA signalling by targeting genes encoding R2R3 MYB transcription factors [38]. According to our small RNA-seq, mdm-miRNA159a expression stayed low during Stages 1 and 2 (endodormancy) and increased during Stages 3 and 4 (ecodormancy) (Figure 2B). This pattern of expression was confirmed by quantifying the pre-miRNA level of mdm-miRNA159a during the dormancy cycle in apple buds (Figure 4A). The mRNA level of its putative target genes *MdMYB33* and *MdMYB65* was also differentially expressed during the dormancy cycle (Figures 3C and 4B,C). As shown in the Figure 4B, *MdMYB33* mRNA was upregulated during endodormancy (between December and January) and strongly downregulated in the ecodormancy (from February to March), coinciding with the maximum accumulation of mdm-mRNA159a pre-miRNA levels (Figure 4A). Although the expression of *MdMYB65* mRNA was similar to that shown for *MYB33* in the years 2016/2017, this pattern was not reproduced in 2018/2019 (Figure 4C). The downregulation of *MdMYB33* and *MdMYB65* expression could be caused by the activity of mdm-miR159a, since these loci contain a canonical miR159 binding site [38] (Figure 4D). Moreover, the miR159a binding site on *MdMYB33* was empirically demonstrated by degradome sequencing [39].

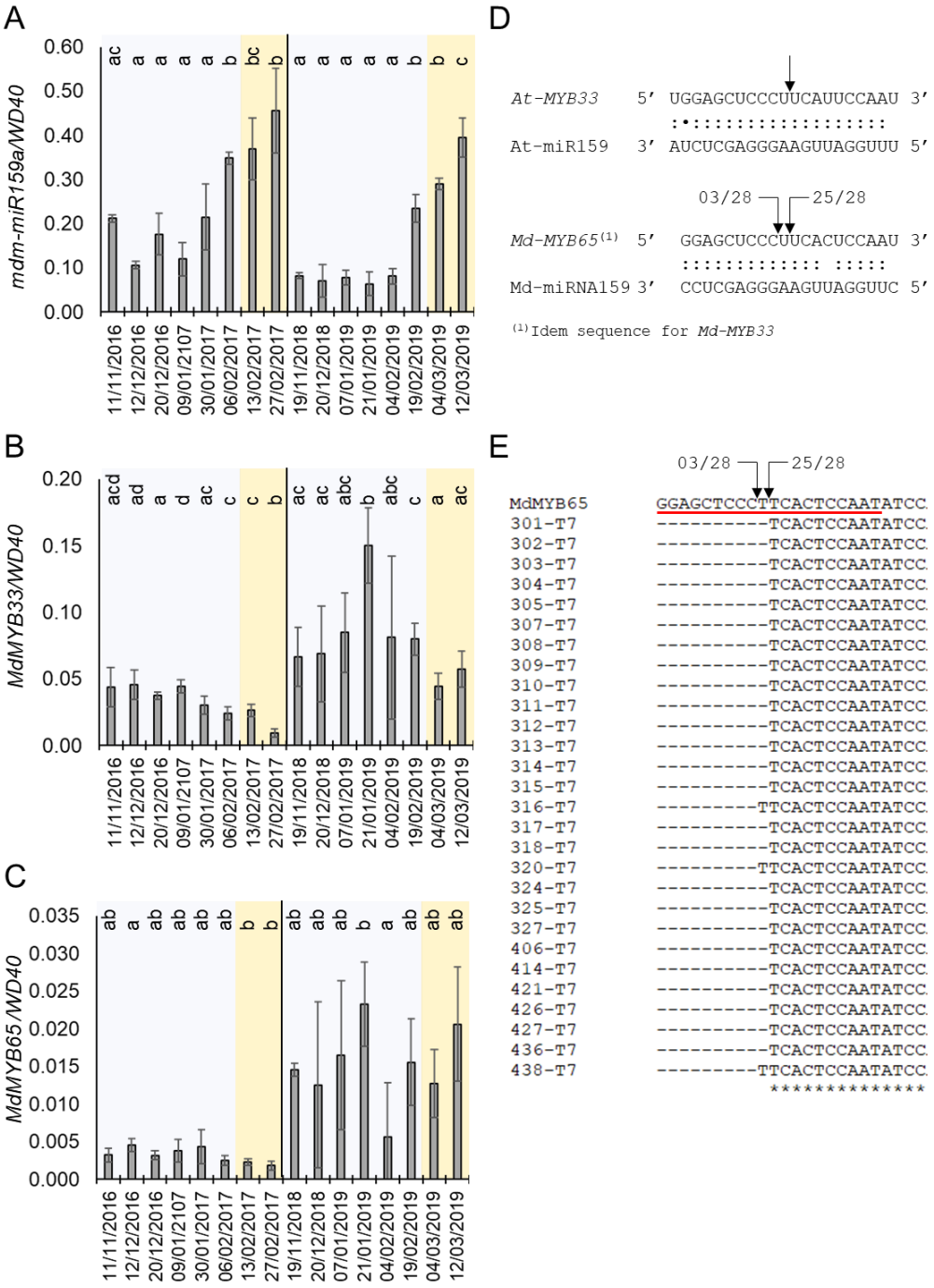

Figure 3-4 Expression of mdm-miR159and their targets during dormancy and in response to ABA. (A) Annual expression profile of pre-mdm-miR159a. (B) Annual expression profile of MdMYB33. (C) Annual expression profile of MdMYB65. (D) Comparison between A. thaliana and apple of target sites of miR159 on MYBs. (E) Alignment of the miR159 target site (red underline) and the sequences recovered from 28 independent clones resulted from the 5′ RLM-RACE assay. In (D) and (E), arrows indicate the 5′ position of the cleaved mRNA fragment identified by RLM-5′ RACE, and the numbers refer to the number of independent clones analyzed. In (A–C), blue and orange shadows mean endodormancy and ecodormancy phases, respectively. Statistical analysis was done using a t-test. Letters shared in common between the dates indicate no significant differences (for $p \leq 0.01$).

Furthermore, we determined the precise mdm-miRNA159a cleavage site on the *MdMYB65* gene by 5′ RNA ligase-mediated rapid amplification of cDNA ends (5′ RLM-RACE). Notably, we detected two potential cleavage sites within the miR159a binding sequence on *MdMYB65* (which is identical to the one in *MdMYB33).* The most frequent cleaved mRNA fragment, supported by 25 out of 28 independent clones, coincides with the same nucleotide sequence that was shown for *A. thaliana* (Figure 4D,C).

The above-mentioned results suggested a role of the mdm-miR159a and its targets *MdMYB33* and *MdMYB65* during dormancy cycle. In *A. thaliana*, miR159a is induced by ABA to reduce the sensitivity to this hormone in a feedback loop that involves the repression of these *MYB* genes [38]. In order to correlate the expression of miRNA159 and the ABA metabolism during dormancy, we monitored the level of expression of ABA biosynthetic and catabolic genes in buds in the same time-course experiment shown in Figure 4. Remarkably, the expression of *MdNCDE1*, a gene that encodes the ABA biosynthetic enzyme 9-cis-epoxycarotenoid dioxygenase [40], was induced at certain dates during endodormancy (winter period) and highly reduced during ecodormancy. This result suggested an ABA biosynthetic activity during the endodormancy phase. Furthermore, the mRNA level of *MdABA8'H*, which encodes an ABA 8′-hydroxylase enzyme involved in ABA catabolism[40], was clearly upregulated during endodormacy and strongly repressed in ecodormancy (Figure 5B). As *MdABA8'H* mRNA expression is induced by ABA [40], together with the *MdNCED1* expression profile (Figure 5A), this result corroborates previous reports proposing that highest ABA levels are found during endodormancy and negatively correlates with bud break in different fruit tree species, including apple, sour cherry, sweet cherry, pear and peach [12,37,41,42]. This result suggested that miR159a is induced by ABA during apple bud dormancy.

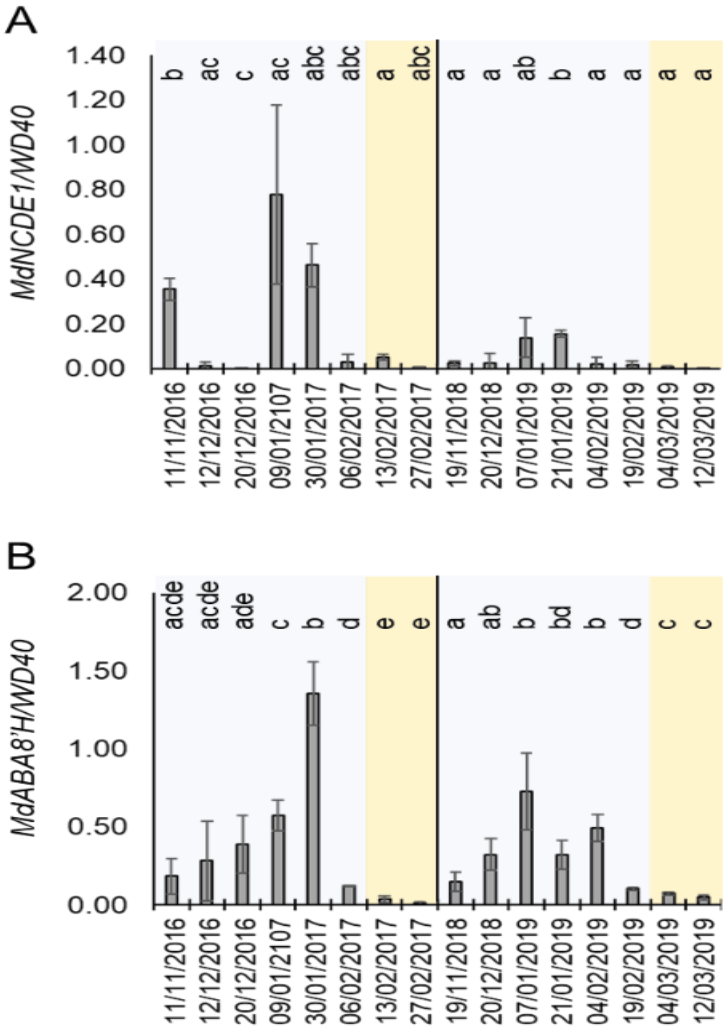

Figure 3-5 Expression of ABA-responsive genes during dormancy. (A) Relative expression of MdNCED1. (B) Relative expression of MdABA8'H. Blue and orange shadows mean endodormancy and ecodormancy phases, respectively. Statistical analysis was done using a t-test. Letters shared in common between the dates indicate no significant differences (for $p \leq 0.01$).

3. Discussion

3.1. Seventeen DE-miRNAs Could Be Part of GRNs Controlling Bud Dormancy in Apple

The transition from endodormancy to ecodormancy and the activation of bud break is believed to be controlled by complex GRNs that integrate endogenous and environmental signals [3,5,43]. TFs are key players in these GRNs, and a timed and coordinated control of their gene expression seems essential to orchestrate dormancy phase transitions [3,5]. This tight regulation is likely to occur not only at the transcriptional level but also post-transcriptionally. MiRNAs are regulatory molecules that exert a large part of their function through the post-transcriptional inhibition of major transcription factors [44]. Furthermore, miRNA can diffuse across tissues and act as long-distance

signals [45] making them central players in developmental GRNs, as those regulating dormancy in trees. The sampling of apical dormant buds from a homogeneous shoot type (spurs) may have favoured the detection of miRNAs, as such buds are synchronized in their developmental stages up to outgrowth. Our small-RNAseq approach and data analysis resulted in the identification of 17 DE-miRNAs possibly involved in GRN controlling bud dormancy of apple. Within these 17 miRNAs, some of them were already found DE during bud dormancy in other tree species. This is the case of members of the miRNA families miR390, miR858, miR164 and miR159 that were also DE during dormancy in tree peony [27] and Japanese pear [26]. However, the observed miRNA expression patterns are not always consistent among species, which suggest a partial evolutionary divergence on the mechanisms controlling dormancy across taxa. Supporting this idea, two recently described miRNAs, miR6390 and miR6285, in white pear [24] and peach [29], respectively, were not detected in apple buds during dormancy. Nevertheless, the 17 DE-miRNAs we identified here are likely to participate in dormancy-related GRNs. Confirming this hypothesis will require a further molecular and functional validation of these miRNAs and their target genes.

3.2. Potential Roles of Target Genes of DE-miRNAs during Bud Dormancy of Apple

In silico prediction identified more than 300 target genes for the 17 DE-miRNAs. The functional classification of these potential target genes based on GO enrichment, allow us to hypothesize on the role of the DE-miRNAs in apple dormancy. Corroborating the expected role of the DE-miRNAs in dormancy-associated GRNs, a large part of the predicted target genes encoded TFs, and in particular MYB TFs, that were included in the GO term of regulation of gene expression. The DE-miRNAs' potential targets were also associated with GO terms related to phenylpropanoid biosynthesis, plant development and growth and response to stimulus. Interestingly, phenylpropanoids are secondary metabolites that include flavonoids, monolignols, phenolic acids, stilbenes and coumarin, and have essential roles in plant development. Their function has been already related to bud dormancy in trees. For instance, changes in the expression of genes involved in phenylpropanoid pathway during bud dormancy were reported in apricot (*Prunus armeniaca*) grapevine (*Vitis* species) and pear (*Pyrus pyrifolia*) [46–49]. Moreover, some flavonoids (e.g., kaempferol and quercetin) have functions as auxin transport and redox scavengers [50–52] and could be involved thus in bud dormancy and growth resumption [53,54]. In *A. thaliana*,

miR858 silences the expression of several MYB TFs involved in the phenylpropanoid pathway, including MYB11, 12, 111and 123 [55]. We found mdm-miR858 and ath-miR858b among the identified 17 DE-miRNAs and both of them were specifically upregulated at the transition between endodormancy and ecodormancy and downregulated in the ecodormancy (Figure 2C). Most of the predicted targets of these two miRNAs were upregulated in ecodormancy and encoded MYB TF related to phenylpropanoid pathway (Figure 3C). This is the case of MD02G1087900, MD08G1070700, MD15G1215400 and MD15G1215500, which are predicted orthologues of the proanthocyanidins biosynthesis activator of *A. thaliana* MYB123 TF [56], and MD15G1215100, MD01G1084400 and MD15G1051000, encoding putative MYB3 TF orthologues, which in *A. thaliana* represses phenylpropanoid biosynthesis gene expression [57]. Thus, the miRNAs mdm-miR858 and ath-miR858b could function as modulators of phenylpropanoid during dormancy. This modulation seems rather complex, since potential targets of mdm-miR858 and ath-miR858b act as either activators or repressors in the phenylpropanoid pathway.

Several DE-miRNA target genes were included in groups of genes enriched for GO terms related to growth and plant development (Figure 3B). A group of these genes encoding NAC (NAM, ATAF and CUC) domain transcription factors are known to play functions related to meristem development in *A. thaliana* and are post-transcriptionally regulated by miR164 [58]. In particular, we found that the apple orthologues of *CUP SHAPED COTYLEDON2* (*CUC2*) (MD11G1253500), *NAC1* (MD10G1198400) and *NAC100* (MD06G1196100, MD09G1053700, MD14G1203000 and MD17G1051600) are potential targets of mdm-miR164c. MiR164 inhibits axillary formation via downregulation of targeted *CUC* genes and it is required for normal branching in *A. thaliana* [59]. The miR164-CUC module seems conserved in other plant species and in cotton (*Gossypium hirsutum*) *gh-miR164* and *gh-CUC2* form a regulatory gene network with *BRANCHED 1* (*BRC1*) that is believed to control branching. *BRC1* encodes a TF that controls branching in several plant species [60,61] and it is part of a GRN controlling dormancy and bud break in apple [5]. Therefore, the downregulation of mdm-miR164c that we observed at ecodormancy (stage 4) (Table 2 and Figure 2C) could be involved in a BRC1-mediated mechanism of bud break control in apple. These data suggest a role of miRNAs in axillary meristem development, bud dormancy and bud break. This notion is supported by the high abundance of the miR166 we observed

in apple buds during endodormancy and ecodormancy (Table S1). In *A. thaliana*, miR166 targets genes encoding HD-ZIP III transcription factors involved in the formation of axillary meristems, root lateral meristems, and determination of lateral organ polarity required for laminar outgrowth [62]. Thus, miRNAs acting in meristem development seem to play a significant role in bud developmental switches of apple.

3.3. A Possible Mechanism of ABA Hyposensibilization Mediated by the Mdm-miR159a-MYB33 Module during Ecodormancy

The plant hormone ABA is a central regulator of dormancy by mechanisms involving repression of cell cycle and intercellular communication via plasmodesmata [52]. In many fruit tree and vine species such as apple, peach, pear, sweet cherry and grapevine (*Vitis vinifera*), ABA levels increase during the endodormancy phase and decline towards bud break [12,36,41,42,53,63]. We could confirm this pattern of ABA accumulation in two consecutive years through the mRNA level quantification of the ABA-responding gene *MdABA8'H* [40] (Figure 5B). Additionally, the pattern of expression of *MdNCDE1* suggests that the observed ABA increase might be triggered by cold temperatures and/or drought typical of winter [40]. In *A. thaliana*, ABA induces the accumulation of miR159, which in turn reduces the sensitivity to this hormone by repressing the expression of *MYB33* and *MYB105*. This feedback loop is essential for ABA homeostasis in plant cells [40]. MiR159 also represses the expression of *MYB65*, which together with *MYB33*, promotes programmed cell death in endosperm and anthers [64]. Notably, we found that mdm-miR159a is upregulated from endodormancy to ecodormancy in apple buds, and that at least one of its potential post-transcriptional targets (*MdMYB33*) is downregulated at ecodormancy. In this context, we speculate that hyposensibilization to ABA by *MdMYB33* downregulation might be required to prevent ABA-mediated growth inhibition at the initiation of bud break and flowering. This could be especially important to attenuate the effect of the rise of ABA levels in growing buds caused by punctual drought and/or cold stress during the springtime. The miR159-mediated repression of *MdMYB33* mRNA is also related to juvenile-to-adult phase transition in *A. thaliana* through the transcriptional control of miR156 and its target gene *SPL9* [65]. However, although we could find small RNAs annotated as miR156 within our sequencing data (Table S1), they were not differentially expressed during the endodormancy to ecodormancy transition, which makes their participation in this biological process unlikely.

4. Materials and Methods

4.1. Plant Material and Experimental Conditions

Apical floral dormant buds from short shoots (spurs) were collected in 2018–2019 winter from *Malus domestica* cv. “Golden Delicious” trees in an orchard located at the experimental station SudExpé in Marsillargues, south of France. All trees were the same age and their phenology appeared tightly synchronized. Four time points were chosen to cover endodormancy to ecodormancy as well as the transition in between (stages 1–4, see Results). For each stage, three biological replicates were collected, each of them being a mix of nine buds from three trees. The dormant stage of the buds was evaluated under forcing conditions (16 h/8 h, light: dark photoperiod at 22 °C) using Tabuenca’s test [32,33].

4.2. RNA Extraction

Total RNA was extracted from ~100 mg bud powder with ‘Spectrum Plant Total RNA Kit’ (Sigma, Darmstadt, Germany) and quantified on a NanoQuant Plate instrument (TTecan, Männedorf, Switzerland). The quality of the RNA was verified in a TapeStation 4200 (Agilent Genomics, Santa Clara, CA, USA) and in an agarose gel. Small RNAs were extracted using Lexogen’s TraPR Small RNA Isolation kit (Lexogen, Wien, Austria) [31].

4.3. Small RNA-seq and Data Analysis

Small RNA libraries were prepared using the Small RNA-Seq Library Prep Kit for Illumina with TraPR (Lexogen) and sequenced on an Illumina HiSeq3000 instrument (1 × 150 bp single read) at the Max Planck Genome Centre in Cologne, Germany. Raw sRNA-seq data was analysed using Mir-Island 2.0 [66]. Reads were mapped to the apple “Golden Delicious” reference genome GDDH13 v1.1 [67]. Plant mature miRNAs from the literature were obtained by combining two miRNA databases: mirBase [68] and PNRD [69], which contain respectively 308 and 208 entries for mature apple miRNAs. Identification of miRNAs within the smal RNA sequencing data was performed as follows: the Mir-Island program first identifies already known small RNAs based on data provided by the user (miRbase and PNRD). Then, it searches for de novo miRNAs by mapping small RNA reads to loci in the genome that can form a

proper RNA hairpin structure that is coherent with a miRNA-producing locus. It also provides the reads of mature miRNA and miRNA* found in the sequencing for each small RNA. An independent miRNA annotation by sequence homology with known MiRNA described in miRBase and PNRD database was also done (Table S1). Mir-Island read counts were then used for differential expression analysis. This was conducted in R 4.0.3, using two common packages for RNA-seq data analysis: EdgeR 3.32.1 [70] and DESeq2 1.30.1 [71]. Counts were normalized using the trimmed mean of M values (TMM) method in EdgeR and the median of ratios method in DESeq2. Low-count tags were discarded according to each package pipeline. In both packages, counts are fitted to Negative Binomial generalized linear models. Fold-changes are estimated and miRNAs are tested for differential expression compared to the first stage (stage 1) with a likelihood ratio test (LRT). False discovery rates (FDR) are calculated using the Benjamini-Hochberg method. Heat maps of DE-miRNAs were constructed using Morpheus (https://software.broadinstitute.org/morpheus, March 2021) and default parameters. RNA secondary structure was calculated using RNAfold [72].

4.4. In Silico Prediction of miRNA Target Genes

MiRNA targets were predicted using the psRNAtarget web interface (2017 release) [73]. We chose a cut-off of E = 3.0 for miRNA-target sequence matching. This only allows to find potential degradation by cleavage, as translation inhibition by miRNAs usually acts when there are more mismatches between the sequence. The “Golden Delicious” reference transcriptome GDDH13 v1.1 was used [67]. Public RNA-seq data [35] were used to build expression heat maps using Morpheus (https://software.broadinstitute.org/morpheus, March 2021) and default parameters.

4.5. Gene Ontology Analyses

The GO enrichment test was done using the Bingo tool (v3.0.3) [74] implemented in Cytoscape (v3.8.2, cytoscape.org, April 2021). The GO term enrichment in the list of 319 potential DE-miRNA target genes was assessed using the hypergeometric test. Raw *p*-values were adjusted for multiple testing using the Benjamin Hochberg method [75]. A high resolution GO map can be found in Figure S1.

4.6. Gene Expression Quantification

RT-qPCRs were performed on a LightCycler 480 using SYBR Green fluorescence detection (Roche, Basel, Switzerland). The parameters used for large were 50 cycles and 60 °C for annealing. PCR efficiencies were estimated using the LinRegPCR program [76]. Primers were designed using Primer3 online interface [77]. Relative quantification was calculated using the ddCT method [78]. WD40 and MDH were used as reference genes [79]. A list of all primers used in this study can be found in Table S5. All statistical analyses were performed using R stats package for R 4.0.3.

4.7. RLM-5′RACE

To validate the predicted miRNA-induced cleavage site, a modified RNA ligase-mediated 5′ Rapid Amplification of cDNA ends was used as described in [80]. From 5 µg of total RNA, the 5′RACE protocol was performed using the GeneRacer kit (Invitrogen Life Technologies, Waltham, MA, USA), according to the supplier's instructions except for the first two steps (decapping and phosphorylation), which are specific of intact transcripts.

In order to maximise amplification specificity, two successive rounds of PCR amplification (nested PCR) were performed using the Phusion High-Fidelity DNA Polymerase with HF buffer (ThermoScientific), primers provided in the GeneRacer kit (GeneRacer 5′ primer and GeneRacer nested 5′ primer in the first and second round, respectively) as forward primers and gene-specific primers (MYB65-GSP and MYB65-nested-GSP) targeting MYB mRNA as reverse primers.

Primer design of the MYB65-GSP and MYB65-nested-GSP primers was performed downsteam of the predicted cleavage site using the Primer3Plus tool (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; September 2021) [77] with the following parameters complying with the recommendations of the GeneRacer kit: amplicon size 70–150 pb, primer length 23–28 nucleotides, primer melting temperature (Tm) 60–74 °C. The sequences of the resulting primers are shown in the Table S5.

4.8. Cloning, Colony PCR and Sequencing

After PCR product separation by gel electrophoresis (1.5% agarose gel in 1× Tris acetate EDTA buffer, migration 100 V/1h30) and visualization under ethidium

bromide/UV light, amplification products with a size compatible with the occurrence of miRNA-directed cleavage were selected. They were purified with the DNA Clean & Concentrator kit (ZymoResearch, Irvine, CA, USA), then their blunt ends were changed into cohesive ends with dATP and Diamond Taq DNA Polymerase (Eurogentec, Seraing, Belgium), thanks to its terminal transferase activity allowing TA cloning. After another purification with the DNA Clean & Concentrator kit (ZymoResearch), cloning was performed using the TOPO-TA Cloning Kit for Sequencing (Invitrogen Life Technologies) following the manufacturer's instructions modified as follows: bacterial transformations were performed using 2.5 µL of ligation reaction with 50 µL of One Shot chemically competent cells. Transformed bacteria (50, 100 or 150 µL) were plated on a Luria-Bertani (LB) medium supplemented with 1.5% agar and with a final concentration of 100 µg/mL ampicillin.

Several produced colonies from each target gene were analyzed by colony PCR with M13 F/R primers followed by electrophoresis on a 1.5% agarose gel. Those of the PCR products displaying a size compatible with the predicted miRNA-directed cleavage were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sanger sequencing of the purified amplification products was performed by Eurofins Genomics (Köln, Germany).

5. Conclusions

Our results revealed that at least 17 miRNAs change their pattern of expression during dormancy in apple buds. Since most plant miRNAs control the activity of multigene TF families, the activity of these 17 miRNAs is expected to have a significant effect on bud development. Thus, the knowledge produced here can pave the way for using miRNAs as a biotechnological tool for improving apple tree traits related to dormancy and bud break. However, this will undoubtedly require a step of miRNA validation and functional characterization that could be done, for example, using gene editing technologies.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Figure S1: A high resolution image of the GO enrichment map shown in Figure 3B. Table S1: List of small RNAs identified in the small RNA-seq

experiment. Table S2: List of detected DE-miRNAs using EdgeR and Deseq2 programs. Table S3: List of potential miRNA target genes identified using psRNAtarget. Table S4: Results of the GO enrichment analysis done on the predicted DE-miRNA target genes. Table S5: List of primers used in this study.

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GENERAL DISCUSSION

Bud dormancy is one of the multiple adaptive mechanisms developed by plants to deal with unfavorable conditions for growth and flowering. Depending on the species, the regulation of dormancy is controlled by distinct environmental cues, such as day-length (e.g., poplar) [1] and temperature (mostly in the *Rosaceae* family) [2]. In apple, the temperature seems to be the main driver that regulates the endodormancy induction as well as the transition to ecodormancy and the activation of bud break. In this Thesis, I have focused on the molecular mechanisms acting during the transition from endo- to eco-dormancy and how they are modulated by environmental and internal cues. I have specifically focused on post-transcriptional mechanisms that are known to operate in the temperature-mediated control of flowering time in the model plant species *A. thaliana* aiming at identifying similar mechanisms involved in dormancy control in apple. In this work, I have dived into the transcriptional regulation of the transition between endo- and eco-dormancy phases and initiated a preliminary exploration of the post-transcriptional regulation of bud dormancy in apple tree.

Using a transcriptomic approach, I observed that there is an important shift in global gene expression when endodormancy is released. The time points used for bud sampling were quite close to each other. Therefore I consider that the resolution of four time-points in six weeks was adequate for screening bud dormancy steps. Between the first and last time points that cover endodormancy to ecodormancy, more than 10.000 genes were differentially expressed ($\log2FC \geq 1$, $FDR \leq 0.05$). This demonstrates a huge reprogramming of gene transcription occurring during the endodormancy release and ecodormancy. My data suggest that the switch between endo- to eco-dormancy is progressive rather than an abrupt change between stages. Indeed, Tabuenca and bud break tests showed a certain variability in chilling hours necessary to release endodormancy in GD and PL cultivars, being especially important for GD. Moreover, several genes were found already transcriptionally activated in the endodormancy stage in response to warm temperatures, some of them having potential functions related to growth promotion. This suggests that there is an overlap between endodormancy and ecodormancy phases. However, this overlap could also result from the pool of buds that were sampled, as they could be at least partly desynchronized in their dormancy phases, possibly due to differences in their within-tree positions and to the variability of micro-climatic conditions. Actually, no non-destructive method exists to precisely check the dormancy stage of a given bud and

mixed buds in pools remains the only possible method so far. In my studies, the cultivars GD and PL, which display contrasted CR and bud break timing, allowed me to identify genes that are potentially related to the dormancy cycle regulation in fruit trees. This was the case of *MdHB22* and *MdANT* genes that could have a role during ecodormancy in the apple tree.

The analyses of gene expression clustering and gene ontology I performed on transcriptomic data have revealed possible pathways being involved in the endo- to eco-dormancy transition. Interestingly, more than half of the genes differentially expressed from endo- to eco-dormancy were up-regulated, mainly related to carbohydrate process, organ development, gene expression regulation and response to hormones. This illustrates a huge transcriptional re-activation to reprogram the shoot meristem in preparation for the growth and reproductive phases. The observed global transcriptomic changes could be contributed by an epigenetic reprogramming conducted by DNA and histone methylation mechanisms, in concordance with the idea that dormancy and vernalization are greatly regulated at the epigenetic level [3,4]. Indeed, several differentially expressed genes during the dormancy cycle were annotated as components of the machinery of gene repression, including a *DICER*-like gene that is potentially involved in the generation of small RNAs and the silencing of *FLC* [5]. On the other hand, genes related to the regulation of response to oxidative stress and to the regulation of transcription were down-regulated in the endo- to eco-dormancy transition. This suggests that redox signaling plays a role in the late endodormancy phase, and it is likely an endodormancy release promoter.

I have also explored the role of AS in the regulation of the dormancy cycle in apple. The bioinformatic pipeline I have implemented was able to identify genome-wide AS events in apple buds during the dormancy cycle. However, it requires further optimization as the isoform annotation was not fully correct. In the meantime, I was able to identify potential isoforms in *MdSVPa* that predominate at different phases during the dormancy cycle. In *A. thaliana*, changes in ambient temperature affect *FLM* AS as part of the thermosensory pathway controlling flowering. Thus, the finding of *MdSVPa* being alternatively spliced in a particular phase of the endodormancy is consistent with the hypothesis that temperature might control the AS machinery during bud dormancy in the apple tree as it does in *A. thaliana* in the regulation of flowering

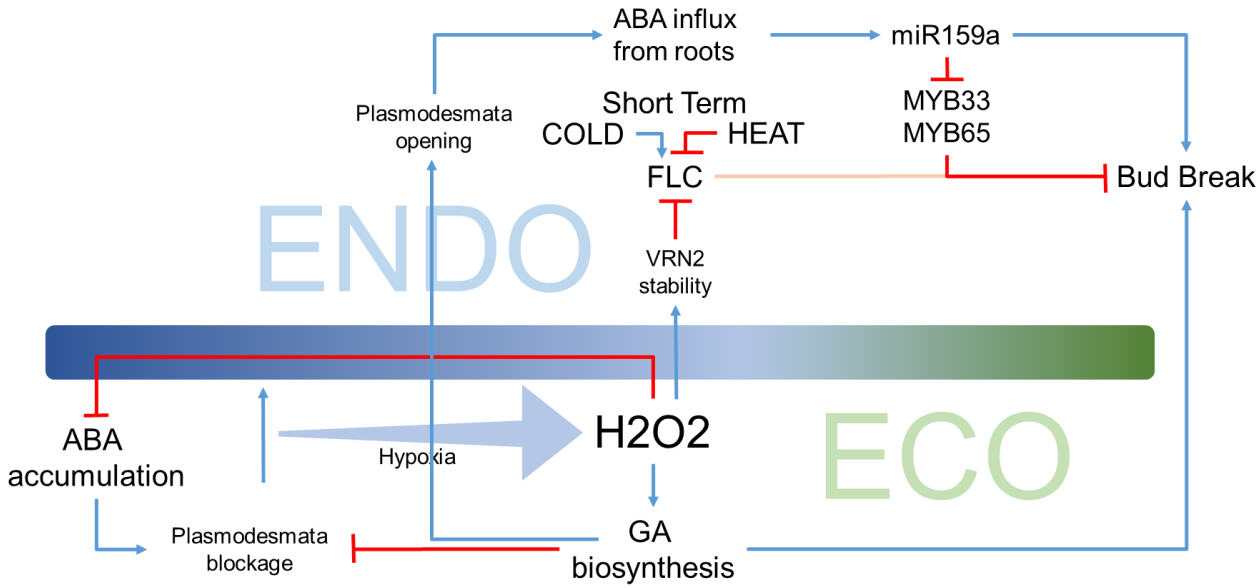
time. Whether the AS observed for *MdSVPa* is also mediated by temperature changes requires further studies.

A major result of this Thesis concerns the possible role of miRNA during the dormancy cycle. To this end, I have studied miRNAs differentially expressed during dormancy phases using a recently developed AGO-purification technique to isolate small RNAs from apple buds. My results revealed that at least 17 miRNAs change their pattern of expression during dormancy in apple buds. These miRNAs could control the activity of several TFs and therefore, their activity is expected to have a significant effect on bud development. Thus, the knowledge produced here can pave the way for using miRNAs as a biotechnological tool for improving apple tree traits related to dormancy and bud break. Furthermore, I have shown the conservation between *A. thaliana* and apple of a regulatory miRNA/target gene module in the context of the ABA homeostasis. This regulatory module could be especially important to attenuate the effect of the rise of ABA levels in growing buds caused by punctual drought and/or cold stress during the springtime.

Even though the post-transcriptional regulation during bud dormancy must be further investigated, this work brings some pieces of knowledge about the role of AS and miRNAs dependent mechanisms in the regulation of endodormancy and ecodormancy. Moreover, our results suggest a possible epigenetic control mediated by siRNAs (e.g., 24 nt found in the small RNA-seq) that could integrate gene regulatory networks controlled by transcription factors and hormone signaling pathways and modulated by the environmental conditions.

The dormancy cycle is regulated by environmental and endogenous cues. Here, we studied the effect of temperature and ROS signaling as potential cues regulating gene expression during dormancy. By applying H_2O_2 to buds during the mid-endodormancy phase, we showed a change in the expression of some genes and phenological differences in bud break percentages. This leads us to propose that ROS could play a role, at least, in the endodormant phase in the signaling for repression of the dormancy machinery and/or as a promoter of the awakening of metabolism, re-starting the cellular activity. By applying warm temperatures, we could identify the genes that are warm-responsive and could be related to bud growth control. It is worth mentioning that several of the studied genes were able to respond to ROS and/or

temperature during endodormancy. The fact that warm temperatures gave rise to transcriptomic changes that are not reflected in bud break acceleration suggests a strong genetic blockage of growth promotion downstream of temperature signaling. However, ROS signaling seems more effective in removing such genetic blockage. This might be caused by the observed premature downregulation of *MdFLC*-like after H_2O_2 treatments. This suggestion needs to be further investigated. Indeed, many discoveries are still to be made in the role of hormonal balance, ROS modulation, temperature perception and their integration in gene regulatory networks controlling dormancy in apple. Also, the influence of other parts of the plant in the bud dormancy set, mainly for the entrance into paradormancy, has not been considered in this work. This stage, before the set of endodormancy, when the plasmodesmata are still open for communication, is not yet well understood. A simplified scheme of the results and the potential hypothesis explaining the molecular mechanisms during bud dormancy progression is proposed below:



Perspectives

Given that this thesis has been developed within the context of global pandemics, some parts of this study remained as preliminary and required further investigation. In particular, my research on the influence of AS in dormancy control is

still preliminary and this part will have to be deeply revisited, reconsidering appropriate methods for analyses. Moreover, the validation of *MdSVPa* isoforms functions, and potentially other isoforms, still needs to be done.

Regarding miRNAs, further studies are still required on their expression patterns through dormancy stages, in combination with that of their target genes. Regarding the group of candidate genes with interesting features based on GO analysis and differential expression, studies allowing to explore their sequences in the diversity of apple genotypes constitute an interesting perspective for the AFEF team. Indeed. Such an exploration of the presence of sequence or allelic variations could take advantage of the presence of the French Core Collection that is installed at the Diascope Experimental Unit in Mauguio (France). This collection is composed of 242 different cultivars that present a wide range of CR and HR. It thus represents a great source of genetic variability that could be considered, in collaboration with other research groups and breeders, for the selection and development of new cultivars with CR and HR characteristics adapted to the future climatic conditions in their cultivation areas.

For all those perspectives, using *A. thaliana* as a plant reference will remain an important strategy. However, deciphering possible new genes or different functions of genes or isoforms involved in the bud dormancy phase in the apple tree but also in the *Rosaceae* family is still challenging. The functional validation of candidate genes in fruit trees using transgenic approaches, such as gene silencing (by RNA interference or mutations) or overexpression, and genome edition are quite difficult and slow if compared to the already established model plants such as *A. thaliana, Oryza sativa* (rice) or *Nicotiana tabacum* (tobacco). Some successful genetic transformations were done in apple, plum and pear, but they are the exception so far. Based on that, the study of our finds in the French Core Collection with its genetic variability is a great resource to overcome that difficulty.

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