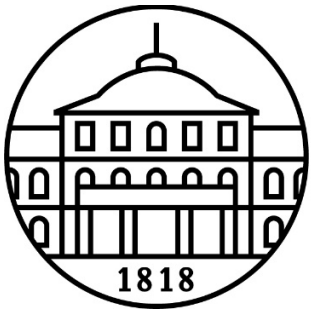

UNIVERSITY OF HOHENHEIM

Faculty of Agricultural Sciences

Institute of Crop Science

Department of Quality of Plant Products

University of Hohenheim

Prof. Dr. Christian Zörb

Physiological, metabolic and molecular basis of biennial bearing in apple

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Julian Kofler

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Examination Committee

Chairperson of the oral examination	Prof. Dr. Ludewig
Supervisor and Reviewer	Prof. Dr. Zörb
Co-Reviewer	Prof. Dr. Flachowsky
Additional examiner	Jun.-Prof. Dr. Schmöckel

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Abbreviations

%	percent
°C	degree celsius
µl	microliter
µm	micrometer
4CL	4-coumaroyl:CoA ligase
Å	Ångström
ABA	abscisic acid
AGAL2	alpha-galactosidase 2
BRR2	BAD RESPONSE TO REFRIGERATION 2
BS	brassinosteroids
C4H	trans-cinnamate 4-monooxygenase
CCOAMT	caffeoyl-CoA O-methyltransferase
CHI	chalcone isomerase
CHS	chalcone synthase
CK	cytokinins
cm	centimeter
CO	CONSTANS
dafb	days after full bloom
DAG	differentially abundant gene
DAM	differentially abundant metabolite
DAP	differentially abundant protein
DFR	dihydroflavonol reductase
DNA	deoxyribonucleic acid
EC	enzyme commission
F3`H and F3`5`H	flavonoid 3` and 3`5` hydroxylase
F3H	naringenin,2-oxoglutarate 3-dioxygenase
FC	fold change
FDR	false discovery rate
FDR	flavan-3,4-diol reductase
FER1	ferritin-1
FLC	FLOWERING LOCUS C
FLS	flavonol synthase
FT	FLOWERING LOCUS T
FWHM	full width half maximum
GA	gibberellic acid
GBSS1	granule-bound starch synthase 1
GDDH13	genome assembly of a 'Golden Delicious' double-haploid tree
GDH	growing degree hours
GO	gene ontology terms
GOF	goodness of fit
ha	hectare
Hd3a	HEADING DATE 3a
HSP	high scoring pairs
Hz	hertz
IAA	indole-3 acetic acid
iTRAQ	isobaric tags for relative and absolute quantitation
JA	jasmonic acid
kDa	kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDOX	leucoanthocyanidin dioxygenase
LEA	late embryogenesis abundant protein
LFQ	label-free quantification
m	meter

M	molar
m/z	mass-to-charge ratio
MCL	Markov clustering algorithm
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
MS	mass spectrometry
Nano-LC-ESI-MS/MS	nanoscale liquid chromatography electrospray ionization tandem mass spectrometry
NCBI	National Center for Biotechnology Center
NGS	next generation sequencing
nl	nanoliter
OMT1	flavone 3'-O-methyltransferase 1
PAL1	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PIP2	phosphatidylinositol 4,5-bisphosphate
PLS-DA	partial least-squares discriminant analysis
QC	quality control
rcf	relative centrifugal force
RNAse	ribonuclease
RNAseq	ribonucleic acid sequencing
RP	reversed-phase
RT	retention time
s	second
SA	salicylic acid
SAMS	S-adenosylmethionine synthetase
t	metric ton
Tb	base temperature
Tc	critical temperature
TCSA	trunk cross-sectional area
TFL1	TERMINAL FLOWER 1
Th	mean hourly temperature
Tu	optimum temperature
UHPLC	ultra-high-performance liquid chromatography

Summary

Alternate or biennial bearing in apple (*Malus ×domestica* Borkh.) is characterized by ‘On’ years with high crop load and inhibited floral bud initiation and ‘Off’ years with little crop load and promoted formation of floral buds, respectively. Apple cultivars differ in their degree of biennial bearing behavior. The cropping irregularity has severe effects on quality and yield of apple harvests in commercial orchards and thereby directly poses an economic risk to apple growers. The aim of this study was to contribute to the understanding of the underlying mechanisms of biennial bearing in apple by analyzing the physiological processes in bud meristems during the time of flower bud induction. A field experiment was conducted during the growing seasons 2015 and 2016 and provided bud meristems of various developmental stages for a variety of analyses. The regular bearing cultivar ‘Royal Gala’ and the biennial bearing cultivar ‘Fuji’ allowed the comparison of two different developmental responses to high and low crop load treatments. Buds from 2-year-old spurs were sampled starting approximately four weeks after full bloom.

Histological analysis of bud meristems successfully identified the time point of flower bud initiation in both cultivars at the experimental site. The onset of flower bud initiation was affected by crop load, cultivar and heat accumulation. While heavy cropping delayed the onset in ‘Royal Gala’ trees for 20 days compared to ‘Royal Gala’ trees with no crop load, bud initiation in heavy cropping ‘Fuji’ trees was negligible. ‘Fuji’ trees with no crop load started initiating buds 19 days earlier than ‘Royal Gala’ trees with the same cropping status.

Proteomic profiling of the buds sampled during flower bud induction and during flower bud initiation revealed distinct differences in specific protein abundances depending on the cropping status. Buds from trees with a high crop load, where the flower bud initiation was inhibited and the buds primarily remained in a vegetative state, showed a decreased abundance of enzymes belonging to the phenylpropanoid and flavonoid pathways. Specifically, PAL was reduced, which could lead to less active auxin due to the reduced production of chlorogenic acid and thereby inhibiting flower bud formation. Furthermore, increased abundances of histone deacetylase and ferritins were also found in buds from high

cropping trees, indicating that histone modification and building up enough iron storage capacities are involved in the vegetative bud development. Buds growing on non-cropping trees with a high rate of flower bud initiation, showed significantly higher concentrations of proteins involved in histone and DNA methylation.

Metabolomic profiling and next-generation RNA sequencing showed that thiamine, chlorogenic acid, and an adenine derivative play a role in metabolic pathways promoting early flower bud development in apple, and that tryptophan was more abundant in buds collected from high-cropping trees compared to non-cropping trees.

The selection of proteins, metabolites, and genes that the current work produced through its broad, non-targeted approach provides a comprehensive data base for future, more targeted analyses. The results of this study lay a thorough baseline to contribute to the identification of biological markers that are linked to a certain bearing behavior. Such markers can accelerate and facilitate breeding programs aimed at selecting apple cultivars, that are less prone to biennial bearing.

Zusammenfassung

Die Ertragsalternanz bei Apfel (*Malus ×domestica* Borkh.) ist gekennzeichnet durch "On"-Jahre mit hohem Ertrag und gehemmter Blütenknospenbildung und "Off"-Jahre mit geringem Ertrag und Bildung vieler Blütenknospen. Verschiedene Apfelsorten weisen eine unterschiedlich starke Ausprägung des Alternanzverhaltens auf. Die Unregelmäßigkeit des Ertrags hat bedeutende Auswirkungen auf die Qualität und Quantität der Apfelernte in kommerziellen Obstanlagen und stellt somit ein direktes wirtschaftliches Risiko für die Apfelanbauern dar.

Ziel dieser Studie war es, durch die Analyse der physiologischen Prozesse in Knospenmeristemen zur Zeit der Blütenknospeninduktion einen Beitrag zum Verständnis der zugrundeliegenden Mechanismen der Alternanz bei Apfel zu leisten. Ein in den Vegetationsperioden 2015 und 2016 durchgeführter Feldversuch lieferte Knospenmeristeme in verschiedenen Entwicklungsstadien für eine Vielzahl von Analysen. Die gleichmäßig tragende Sorte „Royal Gala“ und die stark alternierende Sorte „Fuji“ ermöglichten den Vergleich von zwei unterschiedlichen Entwicklungsreaktionen auf hohe und niedrige Fruchtbehänge. Knospen von 2-jährigen Kurztrieben wurden ab etwa vier Wochen nach Vollblüte beprobt.

Durch eine histologische Analyse der Knospenmeristeme konnte der Zeitpunkt der Knospeninitiation bei beiden Sorten am Versuchsstandort bestimmt werden. Der Beginn der Knospeninitiation wurde durch die Stärke des Fruchtbehangs, die Sorte und die gesammelte Wärmesumme beeinflusst. Während sich der Beginn der Knospeninitiation bei „Royal Gala“ mit starkem Fruchtbehang im Vergleich zu „Royal Gala“ mit niedrigem Fruchtbehang um 20 Tage verzögerte, war die Knospeninitiation bei „Fuji“ mit hohem Fruchtbehang vernachlässigbar. „Fuji"-Bäume ohne Fruchtbehang begannen 19 Tage früher mit der Knospeninitiation als die entsprechenden „Royal Gala"-Bäume.

Proteom-Profile der Knospen, die während der Knospeninduktion und der Knospeninitiation gesammelt wurden, wiesen deutliche Unterschiede in der Häufigkeit spezifischer Proteine in Abhängigkeit vom Fruchtbehang auf. Knospen von Bäumen mit einem hohen Fruchtbehang,

bei denen die Blütenknospenbildung gehemmt war und die Knospen hauptsächlich im vegetativen Zustand verblieben, wiesen eine geringere Abundanz von Enzymen auf, die zu den Phenylpropanoid- und Flavonoid-Stoffwechselwegen gehören. Insbesondere war PAL reduziert, was aufgrund der daraus folgenden verminderten Produktion von Chlorogensäure zu weniger aktivem Auxin führen und damit die Bildung von Blütenknospen hemmen könnte. Darüber hinaus wurden in den Knospen von Bäumen mit hohem Fruchtbehang erhöhte Mengen an Histondeacetylase und Ferritinen gefunden, was darauf hindeutet, dass die Histonmodifikation und der Aufbau ausreichender Eisenspeicherkapazitäten an der Entwicklung der vegetativen Knospen beteiligt sind. Knospen, die an Bäumen mit einer hohen Rate an Blütenknospenbildung wuchsen, wiesen signifikant höhere Konzentrationen von Proteinen auf, die an der Histon- und DNA-Methylierung beteiligt sind.

Metabolomanalysen und RNA-Sequenzierung zeigten, dass Thiamin, Chlorogensäure und ein Adenin-Derivat eine Rolle in den Stoffwechselwegen spielen, die die frühe Entwicklung der Blütenknospen bei Äpfeln fördern, und dass Tryptophan in Knospen von Bäumen mit hohem Fruchtbehang im Vergleich zu Bäumen mit niedrigem Fruchtbehang häufiger vorkam.

Die Auswahl von Proteinen, Metaboliten und Genen, die diese Arbeit durch die breitangelegte Methodik hervorbrachte, bietet eine umfassende Datenbasis für zukünftige, gezieltere Analysen. Die Ergebnisse dieser Studie bilden eine solide Grundlage für die Identifizierung von biologischen Markern, die mit der Ertragsalternanz verbunden sind. Solche Marker können Züchtungsprogramme beschleunigen und erleichtern, die auf die Auswahl von Apfelsorten abzielen, die weniger anfällig für Ertragsalternanz sind.

1. General Introduction

1.1 Apple production and economic importance

Apple (*Malus ×domestica* Borkh.) ranks second in the list of fruit with the highest production quantities around the world with 86,4 million tons in 2020 (Food and Agricultural Organization 2022). Its production quantity in 2020 was surpassed only by bananas with 119,8 million tons. The gross production value of apple in 2018 exceeded 50 billion $, highlighting its economic importance as it represents the second most valuable fruit industry worldwide, surpassed only by grapes with 92 billion $ in 2018 (Food and Agricultural Organization 2022). China accounts for almost half of the total production with 40,5 million tons in 2020. The huge production volume is not necessarily attributed to exceptionally high yields. The average yield of 21 t/ha in China is only slightly above the world average of 18,70 t/ha; but combined with the vast acreage of more than 1,9 million hectares, China dominates the world production of apple. Much higher yields are achieved by intensive apple orchards in countries such as New Zealand (57,42 t/ha), Switzerland (52,62 t/ha) or Chile (59,12 t/ha). Germany ranks on place 13 among the world`s largest apple producers with 1,02 million tons and a yield of 30,12 t/ha in 2020. In Germany, apple contributed 89% to the total tree fruit production in 2021 with the Lake Constance area in the state of Baden-Württemberg and Altes Land in Lower Saxony and Hamburg being the largest production areas, contributing 34,3% and 29% to the apple production in Germany in 2021, respectively (Statistisches Bundesamt 2022). 76,1% of the production was designated for the table fruit market.

The high share of table fruit in the total apple production results in a high demand for constant quality, predominantly determined by size, shape, color, taste and storage quality. Especially a homogenous fruit size is essential for successful marketing, as for example the maximum allowed size deviation for class 1 fruit in the European Union is 5 mm when packed in layers (Amtsblatt der Europäischen Union 2004).

1.2 Factors that determine yield and quality

Fruit growers in commercial orchards aim for constant yields and qualities to be economically profitable and to meet consumer demands. Similar to other pome fruit, crop load in apple is determined primarily by two factors: number and fertility of flowers. As the number of flowers at bud burst limits all further biotic and abiotic influences and horticultural practices that affect yield and quality, an adequate number of fertile flowers is essential. In intensive apple orchards with high crop loads, 3,000 to 4,000 flowers per tree are required to obtain 250 to 300 fruit per tree (Breen et al. 2016).

The reproductive cycle in perennial fruit trees such as apple starts early in the previous year of the actual fruit yield with the induction of flower buds. Buds are induced primarily on two-year-old spurs but also terminally or laterally on one-year-old shoots depending on cultivar, age and growth of the tree (Buban and Faust 1982). Numerous factors influence not only the starting point and duration of flower bud induction, but also the within tree synchronization of flower bud induction, resulting in differently advanced bud development stages on the same tree (Koutinas et al. 2010). The total share of induced buds also depends on various factors. The induction of flower buds overlaps with the development of this year`s young fruit. Studies show that developing fruit inhibit the induction of nearby bud meristems leading to poor return bloom in the subsequent season (Monselise and Goldschmidt 1982; Wünsche and Ferguson 2005a; Wilkie et al. 2008; Schmidt et al. 2009; Samuoliene et al. 2016). While the specific mechanism of this inhibitory effect is still unclear, it is assumed that mobile signals formed by young seeds within the fruit, such as plant hormones like gibberellins (Hoad 1978; Marino and Greene 1981; McArtney 1994), or the lack of certain nutrients like carbohydrates (Monselise and Goldschmidt 1982) play an essential role. It is widely documented, that excessive fruit set has an inhibitory effect on flower induction in the current year, leading to low or no flowering in the subsequent spring (Wünsche and Ferguson 2005a). The resulting bearing behavior, characterized by large yields of small-sized fruit in ‘on’ years and low yields of over-sized fruit in ‘off’ years is termed biennial or alternate bearing (Jonkers 1979; Monselise and Goldschmidt 1982). Usually, apple trees in commercial plantations produce far more flowers than needed

for a commercial crop load (Wertheim and Webster 2005). Thus, flower thinning is routinely practiced in commercial orchards to assure high fruit quality and regular bearing each year (Breen et al. 2016). Preventing a too excessive crop is the first step in controlling biennial bearing (Jonkers 1979). It is important to thin early before the seeds in young developing fruit can inhibit the formation of flower buds. While in most conventional plantations chemical thinning was practiced commonly (Jonkers 1979), mechanical flower thinning has also been made possible recently (Damerow et al. 2007).

Cultivars that produce a large excess of flowers and are not equipped with an efficient self-thinning mechanism, tend to be more prone to alternate bearing, as they have to handle an overload of fruit and thereby trigger the alternate bearing behavior (Monselise and Goldschmidt 1982). The cultivar ‘Fuji’ is such a cultivar, with vigorous growth and a strong tendency to bear biennially (Hampson and Kemp 2003). A more regular bearing cultivar is ‘Royal Gala’, which is not prone to biennial bearing and bears prolific annual crops (Hampson and Kemp 2003).

The induction of floral buds is followed by the bud initiation and differentiation phase, which is temporarily interrupted by the onset of bud dormancy in autumn, and is completed in the subsequent spring shortly before bud burst (Buban and Faust 1982; Tromp 2000; Wilkie et al. 2008). During bud dormancy, the bud goes through different phases, which are called para-, endo- and ecodormancy (Lang 1987). Paradormancy, which is synonymous with apical dominance, commences in fruit trees late in the growing season at the end of summer. The plant hormone auxin, produced in shoot apices, inhibits lateral bud growth (Faust et al. 1995; Horvath 2010). After leaf abscission, fruit trees such as apple enter endodormancy to withstand cold temperatures during winter. Endodormancy begins in autumn and prevents bud growth even if they are not inhibited by apical dominance and environmental conditions are conducive to growth, which prevents bud growth during short periods of warm weather during early winter (Horvath 2010). Cold temperatures induce bud endodormancy, and a certain number of chill units, which varies between cultivars, is required to fulfill the plants chill requirement. When the chill requirement is fulfilled, the buds transition to the next phase of dormancy: ecodormancy (Bubán and Faust 1995; Yu et al. 2020). During ecodormancy, buds regain

competency to respond to external environmental factors, such as the accumulation of heat. The accumulation of heat during ecodormancy is generally considered to be the main driving factor for breaking ecodormancy (Guo et al. 2014). Once the heat requirement is met, the buds can quickly progress toward budburst when favorable conditions are present (Yu et al. 2020). Consequently, a pre-requisite for a high number of fertile flowers is the regular occurrence of certain abiotic environmental conditions with adequate chilling during endodormancy and the accumulation of heat during ecodormancy being among the most prominent ones.

1.3 Apple production and climate change

Due to global climate change, surface temperature is projected to rise and heat waves and extreme weather events will occur more often (IPCC 2015). As a direct consequence, rates of chilling and heat accumulation, which are especially vital for flowering of fruit trees in temperate regions, can be compromised and possibly lead to production losses (Guo et al. 2014).

To complete endodormancy, fruit trees require a certain number of chill units. Projections have already shown dramatic losses in the number of chilling hours in California, Australia, South Africa and most warm growing regions (Luedeling 2012). Future projections also indicate delays of flowering dates of major apple cultivars in apple growing regions in Spain due to insufficient chilling leading to potential production losses (Funes et al. 2016). Symptoms of inadequate chilling range from delayed and poor bud break, a prolonged flowering period, a low proportion of flowering spurs to poor lateral leaf-bud development (Palmer et al. 2003). The decrease in winter chill, which not only reduces apple yields (Luedeling 2012), also affects the starting date of flowering in major apple growing regions in Germany (Pfleiderer et al. 2019).

However, the greater concern is the effect global warming has on the accumulation of heat during ecodormancy. Warmer winters will fulfill the buds` heat requirement during ecodormancy too fast, leading to an early bud burst (Lempe 2020). The advanced flowering date increases the risk of frost damage due to spring frosts occurring after full bloom (Pfleiderer

et al. 2019). Once dormancy is broken in spring, the newly formed flowers are very sensitive to low temperature and can be damaged by spring frosts, leading to severe losses for commercial orchards. During the full bloom stage, ambient air temperatures of -3,9°C can lead to 90% of flowers destroyed (Palmer et al. 2003). Frost damage has not only the potential to wipe out the current year`s crop completely, but also to trigger alternate bearing behavior (Monselise and Goldschmidt 1982).

Global warming accompanied by increased winter temperatures in most temperate regions, which are the most important apple growing regions, will present major challenges for commercial apple growers.

1.4 The need for regular bearing apple cultivars

Breeding so called ‘climate-smart’ cultivars (Salama et al. 2021) whose chill and heat accumulation requirements for bud break can be adapted precisely to regional environmental conditions is essential to equip farmers with resilient apple cultivars to ensure productivity in future everchanging climatic conditions. Regular bearing cultivars that are less prone to show a biennial bearing behavior and that develop a consistently high number of fertile flowers every season, can contribute to achieving regular, high crop loads. A pre-requisite for breeding such cultivars is to fully understand the process that is at the center of the complex reproductive cycle in apple: flower induction. However, the knowledge of the mechanism of flower induction and bud formation in apple is still lacking (Wertheim and Webster 2005; Bangerth 2006).

1.5 Knowledge gap: from understanding flower induction to biomarkers

The signal, that triggers the flower bud development during flower induction, the first stage in the 2-year reproductive cycle of apple, is still unknown (Buban and Faust 1982; Hanke et al. 2007; Hättasch et al. 2008; Koutinas et al. 2010). At some time point in spring, the vegetative meristems perceive a specific signal, that either promotes flower bud development by triggering various processes or suppresses factors that cause the meristem to remain in a

vegetative state (Dolega and Link 2002). However, the exact time point of flower induction seems to be an open issue in many fruit trees such as apple (Smith and Samach 2013; Breen et al. 2016). It is, however, possible, to precisely identify the time point of the following flower bud initiation due to the distinct morphological changes that occur in the bud`s cell structure during that phase (Hanke 1981; Foster et al. 2003; Hoover et al. 2004). Based on mRNA expression data of flowering genes in apple, it can be assumed that flower induction occurs roughly two weeks prior to flower initiation (Hanke et al. 2007). Consequently, determining the time point of flower bud initiation is crucial to select the relevant bud meristems for analyses aiming at understanding the underlying mechanisms of flower bud induction. Furthermore, a major constraint to obtaining robust data about flowering related physiological processes in bud meristems, is the fact, that usually a mixture of induced and non-induced buds is sampled. Such a composite sample of buds with different developmental stages introduces an error to the dataset. Consequently, an experimental setup is required, that reduces the probability of sampling a mixture of induced and non-induced buds. It is widely documented, that heavy cropping decreases or inhibits flower initiation (Wünsche and Ferguson 2005a). Hence, it is necessary to artificially set high and low crop loads to assure that as many buds as possible either remain vegetative or commit to flower formation.

Recent advances in high-throughput omics approaches such as transcriptomics, proteomics and metabolomics allow a more holistic understanding of biological processes. The aim of the current study is to apply these state-of-the-art omics technologies in order to understand the underlying physiological processes in flower bud development in apple. More specifically, the focus is set on processes on the transcriptomic, proteomic and metabolomic level in bud meristems during the first phase of flower bud development, the flower induction phase. Understanding the physiological processes might allow to identify biological markers that can possibly be linked to phenotypes that regularly produce high numbers of fertile flowers are less prone to biennial bearing. Such biomarkers would allow breeders to equip commercial apple growers with resilient, high-cropping cultivars, that are prepared to withstand future ecological challenges due to global climate change.

1.6 Objectives

The objectives of this study are:

1. Determine the time point of flower bud initiation in a regular and a biennial bearing apple cultivar through histological analysis to allow further transcriptomic, proteomic and metabolomic analysis of processes occurring in the bud meristem during flower induction, approximately two weeks prior to the determined flower initiation phase.
2. Identify differences in the proteomic profile of vegetative and floral buds and between buds from a regular and a biennial bearing apple cultivar to contribute to the identification of biological markers that are linked to a certain bearing behavior.
3. Integrate transcriptomic, proteomic and metabolomic data from vegetative and floral buds sampled from a regular and a biennial bearing cultivar to outline holistic gene-to-product pathways possibly explaining cultivar differences in flowering behavior.

2. High Crop Load and Low Temperature Delay the Onset of Bud Initiation in Apple

scientific reports

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Julian Kofler[1], Anton Milyaev[1], Filippo Capezzone[2], Slobodan Stojnić[3], Nikola Mićić[3], Henryk Flachowsky[4], Magda-Viola Hanke[4], Jens-Norbert Wünsche[1*]
[1]Institute of Crop Science, Dept. of Crop Physiology of Specialty Crops (340f), University of Hohenheim, Stuttgart, Germany
[2]Institute of Crop Science, Dept. of Biostatistics (340c), University of Hohenheim, Stuttgart, Germany
[3]Faculty of Agriculture, University of Banja Luka, Banja Luka, Bosnia and Herzegovina
[4]Institute for Breeding Research on Fruit Crops, Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Dresden, Germany

Abstract
The reproductive cycle of apple (*Malus ×domestica* Borkh.) starts with the induction of floral development, however, first morphological changes within the bud appear during the following period of bud initiation. This study identifies the onset and duration of bud initiation in the apple cultivars 'Fuji' and 'Gala', characterized by biennial and non-biennial bearing behaviour, respectively, and describes the effect of crop load and heat accumulation on the temporal pattern of floral development.
The onset of flower bud initiation in heavy cropping 'Gala' trees was delayed for 20 days compared to trees with no crop load, but the rate of initiation was not affected by crop load. Bud initiation on heavy cropping 'Fuji' trees was minor, whereas trees with no crop load started initiating buds 19 days earlier than those of 'Gala' despite the same cropping status and growing degree hours in a given year. The onset of bud initiation in 'Fuji' 'off' trees was 5 and 20 days after summer solstice, respectively, in two consecutive growing seasons, suggesting that this process is driven by heat accumulation rather than by daylength.
The results indicate, that the genetic make-up of the cultivar determines the onset of bud initiation. This can be delayed by increasing crop loads and low temperatures at the beginning of the flower formation process.

Keywords: alternate bearing, bud meristem, flower induction, histology, logistic regression

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2.1 Introduction

The two key factors determining crop load in apple (*Malus ×domestica* Borkh.) are flower density and fruit set (Hanke et al. 2007). Since fruit set can be adjusted to some extent by flower or fruitlet thinning, the critical factor is the number of flower buds per tree (Dennis 2003). Fruit growers aim for stable numbers of flower buds across years to reduce the risk of triggering the phenomenon of biennial bearing (Jonkers 1979). This cropping irregularity is characterized by large yields of small-sized fruit in 'on' years and low yields of over-sized fruit in 'off' years (Williams and Edgerton 1981), i.e. trees with 'on'-bearing status change to 'off'-bearing status in the subsequent year and vice versa. Apple cultivars differ in their degree of biennial bearing behaviour, e.g. 'Gala' has a regular bearing habit, whereas 'Fuji' shows a strong tendency to bear biennially (Hampson and Kemp 2003). Besides apple, biennial or alternate bearing is also commonly found in other fruit trees such as pistachio, pecan, olive, citrus, avocado or mango (Monselise and Goldschmidt 1982). The physiological reason for entering an 'off' year is supposedly the competitive overlap of flower bud formation for the subsequent season and fruit development during the current season (Pratt 1988; Dennis and Neilsen 1999).

Perennial fruit trees such as apple start their reproductive development early in the first growing season of a 2-year-cycle with inducing flower buds at the first stage of the flower bud formation process (Buban and Faust 1982). During this stage, termed flower induction, the vegetative meristem perceives a specific signal that either promotes flower bud development by triggering various biochemical processes or suppresses factors that cause the meristem to remain in a vegetative state (Dolega and Link 2002). For example, as recently shown (Xing et al. 2015a), sucrose could act as a signal molecule for mediating flower induction in apple. Several studies reported that high crop load inhibits flower induction in apple, leading to poor return bloom in the following year (Monselise and Goldschmidt 1982; Wünsche and Ferguson 2005a; Wilkie et al. 2008; Schmidt et al. 2009; Samuoliene et al. 2016). The exact mechanism of crop load-induced inhibition of flower induction still remains unclear, although there is good evidence that a high yield reduces next year`s flower density, hence crop load, in a specific way: mobile signals formed by developing fruit or specifically seed within the fruit (e.g. plant hormones such

as gibberellins) (Hoad 1978; Marino and Greene 1981; McArtney 1994) or lack of certain nutrients (e.g. carbohydrates) (Monselise and Goldschmidt 1982) inhibit the nearby bud meristem. Moreover, high fruit load and high levels of gibberellins are accompanied by an increased expression of the *TFL* gene (Samach and Smith 2013; Haberman et al. 2016) and reduced expression of *FT* in apical buds (Kittikorn et al. 2011) during flower induction.

At microscopic scale, the first visible structural changes of the bud meristem appear during the second phase, the flower initiation. A pronounced doming and broadening of the bud apex is the first sign of floral commitment and marks the developmental onset of floral structures (Foster et al. 2003; Hanke et al. 2007). However, bud initiation within a given tree is synchronized to a certain extent by internal regulatory mechanisms and reported to persist for three to up to seven weeks (McArtney et al. 2001; Dolega and Link 2002).

The subsequent flower bud differentiation, the third stage of the flower formation process, refers to the development of inflorescence primordia and floral organs and is temporarily interrupted with the onset of bud dormancy (Wilkie et al. 2008). The formation of pollen sacs and ovules completes the bud differentiation in the following spring shortly before bud burst (Tromp 2000).

Studying flower bud induction in apple has two main constraints. First, only time-dependent analyses of the bud cell structure provide a precise starting point of doming of the bud meristem, thus the transition from vegetative to floral meristems. Samples collected prior to this time point are then useful for studying the stage of flower induction. Second, composite samples of induced and non-induced buds are typically collected, yet this within-tree inhomogeneity introduces an experimental error for studying the underlying mechanisms of flower bud induction. However, by choosing extreme crop loads of heavy and no fruiting, there is a greater probability that all buds either remain vegetative or commit to flower formation. Consequently, in the current study, apple buds were collected from a biennial and non-biennial bearing apple cultivar with heavy and no crop, respectively, over two growing seasons and microscopically evaluated for determining the onset and duration of flower bud initiation in relation to heat accumulation. The results will define the starting point for RNAseq, proteome

and metabolome analyses of the sampled apple buds prior to the identified flower initiation period. Moreover, we evaluated whether the reduction in return bloom following a high cropping year is the result of a delayed flower bud initiation and thus an incomplete flower bud development or fewer initiated buds in the preceding season.

2.2 Materials and methods

2.2.1 Location and environmental conditions

Field experiments were conducted during the growing seasons in 2015 and 2016 at the Centre of Competence for Fruit Cultivation near Ravensburg, Germany (47°46'2.89"N 9°33'21.21"E, altitude 490m). Climatic conditions in both years were typical for this fruit growing region. To evaluate the effect of temperature on flower bud initiation, the accumulated heat sum was calculated using the concept of Growing Degree Hours (Anderson et al. 1986; Luedeling et al. 2009) (GDH), using the following two equations:

$$\boldsymbol{GDH = F\frac{T_u - T_b}{2}\left(1 + \cos\left(\pi + \pi * \frac{T_h - T_b}{T_u - T_b}\right)\right)} \quad (1)$$

$$\boldsymbol{GDH = F(T_u - T_b)\left(1 + \cos\left(\frac{\pi}{2} + \frac{\pi}{2} * \frac{T_h - T_u}{T_c - T_u}\right)\right)} \quad (2)$$

where T_h is the mean hourly temperature, T_b is the base temperature, T_u is the optimum temperature, T_c is the critical temperature and F is a plant stress factor that was set in both equations to 1. T_b, T_u and T_c were set to 4°C, 25°C and 36°C as suggested for fruit trees (Anderson et al. 1986). Equation 1 was used for mean hourly temperatures between T_b and T_u, equation 2 was used for mean hourly temperatures between T_u and T_c.

Figure 1 shows the accumulated GDH with a base temperature of 4°C calculated from the beginning of the year. From full bloom until the end of the experimental period at 18 weeks after full bloom, 2015 was with 37192 GDH warmer than 2016 with 36309 GDH.

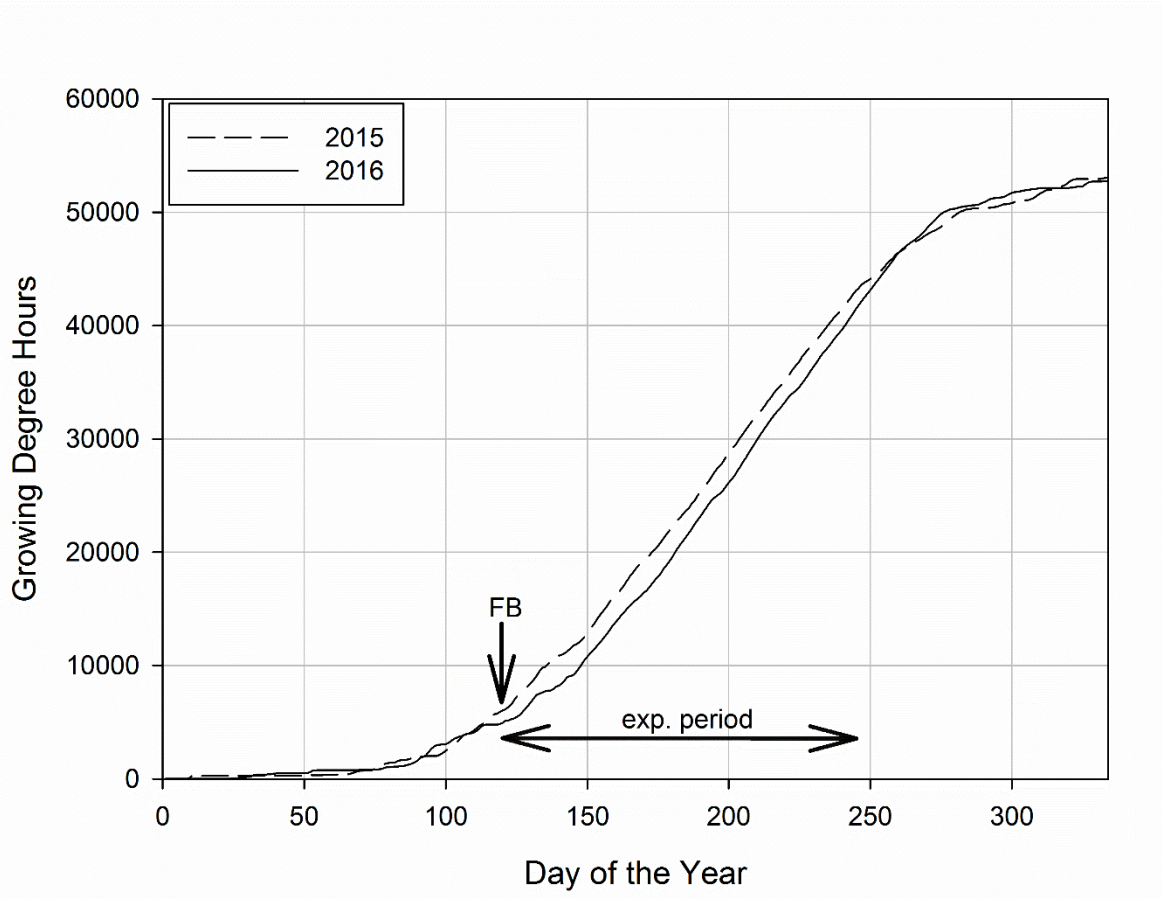

Figure 1 Growing degree hours (GDH) with a base temperature of 4°C, accumulated from the beginning of each year. The experimental period started at full bloom (FB) and continued for 18 weeks.

2.2.2 Experimental design

In this study, two apple cultivars with a different degree of biennial bearing behaviour were used. 'Fuji', clone 'Raku-Raku', is known to be a strongly biennial bearing cultivar, whereas 'Gala', clone 'Galaxy', is considered to bear more regularly. Both cultivars were grafted on M9 rootstock, planted in 2009 and trained as tall spindles of 4.5 m height. Standard orchard management practices, including winter pruning and fertilizer and pesticide applications, followed best practice guidelines. Date of full bloom, defined as the time point when at least 80% of all flowers are fully open, was 30 April 2015 and 27 April 2016 for both cultivars. This was determined by counting the number of open and closed flowers on selected branches of each cultivar. The experiment was carried out in two rows of 'Fuji' adjacent to two rows of 'Gala' with a tree spacing between rows of 3 m and within rows of 1 m. Trees were adjusted to commercial crop loads by mechanical and chemical thinning practices in previous years. At

full bloom, 130 trees per cultivar were selected based on their flowering status within the two rows: 65 trees with a cultivar-specific low bloom density and 65 trees with a high bloom density, respectively. In 2015, the trees with low bloom density received the ‘off’ treatment by manually removing all flowers at full bloom, whereas trees with high bloom density received the ‘on’ treatment and were not flower-thinned. In 2016, the experimental trees were assigned to the opposite cropping treatment of the previous season to maintain the biennial bearing pattern, i.e. trees that were ‘off’ had all remaining flowers removed at full bloom and trees that were ‘on’ were not flower-thinned. Bloom density was expressed as number of flower clusters per trunk cross-sectional area (TCSA), calculated from the trunk circumference measured just prior to full bloom at 20 cm above the graft union (Samuoliene et al. 2016).

2.2.3 Bud sampling

In 2015, bud sampling started in the fourth week after full bloom and continued for 15 weeks. Histological analysis of these buds indicated that bud initiation did not start before the eleventh sample was taken at 99 dafb. We therefore decided to shift the sampling period towards the seventh week after full bloom in 2016 and to shorten the sampling period to eight continuous weeks that should include both the stage of flower induction and the onset of flower initiation. An additional sample was taken 18 weeks after full bloom in 2016 to assess the bud initiation status near the end of the growing season.

At each sampling date, a new set of three trees per cultivar and treatment was randomly chosen and buds from 2-year-old spurs (≤ 5 cm) were collected from the periphery of the tree canopy. Sample size was one bud from each of three trees per cultivar and treatment at each sampling date whereas two buds from each of three trees per cultivar and treatment were taken for the last sample in 2016. In total 300 buds were collected for microscopy analysis. The tissue was immediately fixed in FAA (Foster et al. 2003) (3.7 % formaldehyde, 5 % acetic acid, 50 % ethanol) and stored at 4°C until analysis.

2.2.4 Bud microscopy

Buds were first dehydrated in a graded ethanol and xylol series and then infiltrated with paraffin at 58°C. Each bud was first trimmed to one side before 100 median longitudinal sections of 5 µm thickness were prepared and stained with Wacker`s trichromatic W-3A botanical stain, containing acridine red, acriflavine and astra blue.

Consequently, 30,000 sections were prepared for determining ‘doming’ by selecting the centre of the meristem based on the parallel alignment of cells in the outer three tunica cell layers of the meristematic tissue. As shown in Figure 2, the centre of the meristem was found in nearly all 100 sections, indicating that this detailed microscopy analysis is needed for detecting initiated buds.

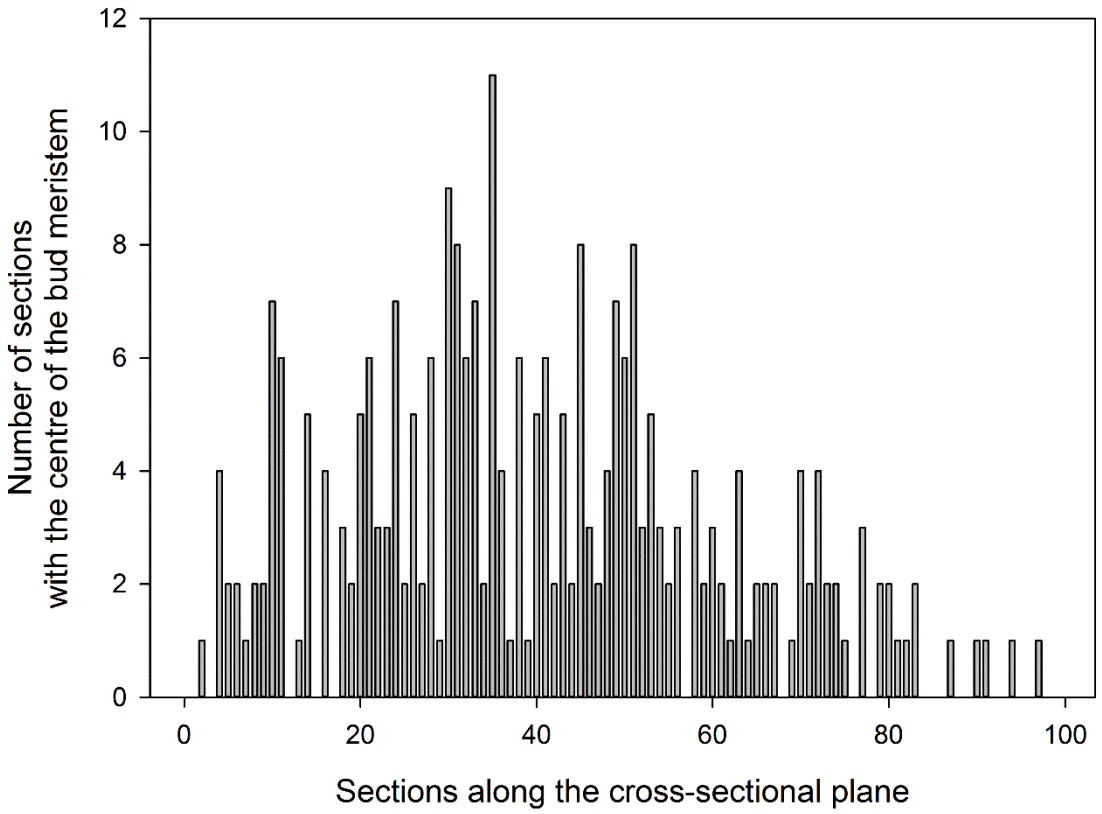

Figure 2 Number of sections that represented the centre of the bud meristem for each section along the cross-sectional plane (n = 268 buds).

Bud developmental stages were determined following the classification (Hanke 1981) shown in Figure 3.

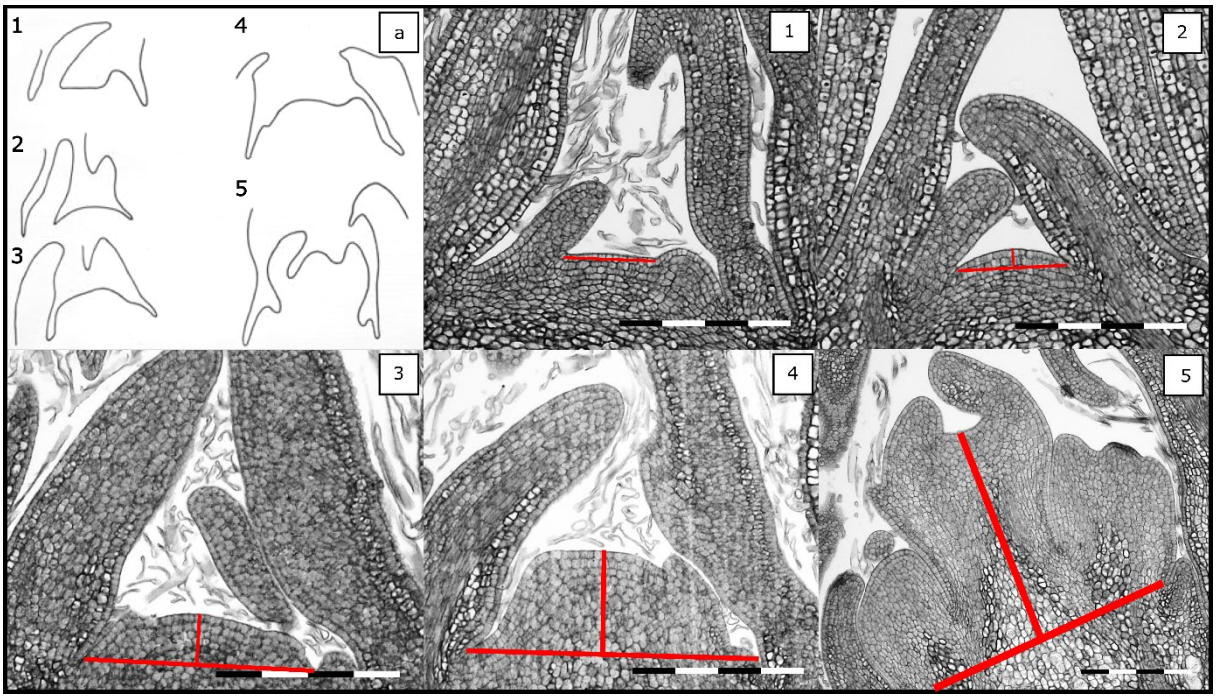

Figure 3 Five apple bud developmental stages. (a) Schematic representation of the five stages of floral bud development as previously shown in literature (Hanke et al. 2007); (1) narrow and flat vegetative apical meristem; (2) broad and swollen vegetative meristem; (3) doming of the apex as the first morphological sign of floral initiation; (4) formation of the inflorescence primordia; (5) differentiation of the inflorescence. Stages 1 and 2 are regarded as non-initiated, whereas stages 3 to 5 are considered as initiated. Full bar = 200 µm. Red lines indicate diameter and height of the meristem at each stage.

Developmental stages one and two were considered ‘non-initiated’ since it is not possible to determine microscopically whether the floral pathway has been triggered. In contrast, stages three to five were considered ‘initiated’ since the morphological characteristics of the meristems clearly indicate that floral development has started. The following binary dataset was used to model the probability of bud initiation in relation to heat accumulation. Furthermore, diameter and height of the bud apex were measured as shown in Figure 3, using the image processing software ImageJ 1.50g.

2.2.5 Modelling the probability of bud initiation

Since the cultivars were not randomly allocated within the orchard block, trees per cultivar were considered as subsamples rather than true replicates. Consequently, no models were fitted with the factor cultivar since this main effect would have been confounded with position effects within the orchard. Accordingly, separate models for each cultivar were used to estimate the probability of buds being ‘initiated’ within a given sample in relation to heat accumulation since

full bloom, using a logistic regression where the logarithm of the odds follows a linear model (Agresti 2007).

The model for the cultivar 'Gala' (1) was only fitted to the 2015 data since bud initiation occurred after the last sampling date at 99 dafb in 2016:

$$\log\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = \mu + \alpha_j + \beta_1 x_i + \beta_{2j} x_i \quad (1)$$

where π_{ij} denotes the probability of a bud being initiated at the i-th gdh of the j-th treatment; μ is the overall intercept; α_j represents the treatment-specific deviation from the common intercept for the *j*-th treatment; β_1 is the common slope of the regression on growing degree hours accumulated from full bloom (gdh, x_i); β_{2j} is the treatment-specific deviation from the common slope for the *j*-th treatment.

The model for the cultivar 'Fuji' (2) was fitted to the 'off' treatment since only one initiated bud was found in 'on' trees; thus no information was available to describe the bud initiation period in 'on' trees:

$$\log\left(\frac{\pi_{im}}{1-\pi_{im}}\right) = \mu + \gamma_m + \beta_3 x_i + \beta_{4m} x_i \quad (2)$$

where γ_m is the deviation from the common intercept μ for year *m*; β_3 is the common slope of the regression on growing degree hours; β_{4m} is the year-specific deviation from the common slope for the m-th year.

Model terms were tested for significance by partial Wald-type χ^2-tests. Throughout the entire statistical analysis, a significance level of $\alpha = 5\%$ was used and non-significant interaction terms that indicated a common slope were removed from the model, following the common convention for keeping a model as simple as possible and to ease interpretation (Agresti 2007).

For better visualization of the results, the model equations were back transformed from the logit-scale to the scale of response probabilities π_{ij} (3), as shown exemplarily for 'Gala':

$$\pi_{ij}(x) = \frac{exp\,(\mu+\alpha_j+\beta_1 x_i+\beta_{2j} x_i)}{1+exp\,(\mu+\alpha_j+\beta_1 x_i+\beta_{2j} x_i)} \quad (3)$$

To obtain the rate of initiation as a coefficient that describes the change of the probability per unit of heat accumulation, the response probability function was differentiated with respect to x_i (4), as shown exemplarily for 'Gala':

$$\frac{\mathbf{d}\pi_{ij}}{\mathbf{d}x_i} = \left(\boldsymbol{\beta} * \left(\frac{exp\,(\mu+\alpha_j+\beta_1 x_i+\beta_2 x_i)}{1+exp\,(\mu+\alpha_j+\beta_1 x_i+\beta_2 x_i)}\right)\right) * \left(\mathbf{1} - \left(\frac{exp\,(\mu+\alpha_j+\beta_1 x_i+\beta_2 x_i)}{1+exp\,(\mu+\alpha_j+\beta_1 x_i+\beta_2 x_i)}\right)\right) \quad (4)$$

Equations (3) and (4) were also considered for 'Fuji' according to the model described in equation (2).

Logistic regression modelling was done using the LOGISTIC procedure in the software package SAS 9.4 (SAS Institute, Cary, USA).

2.3 Results

Return bloom in 2016 was considerably greater on trees that were ‘off’ compared to ‘on’ in 2015 (Fig. 4). ‘Fuji’ ‘off’ trees had 35-fold more flower clusters per trunk cross sectional area than ‘Fuji’ ‘on’ trees, whereas ‘Gala’ ‘off’ trees carried only 5 times more flower clusters than ‘Gala’ ‘on’ trees. The return bloom in 2017 was rather similar to that found in 2016 (data not shown).

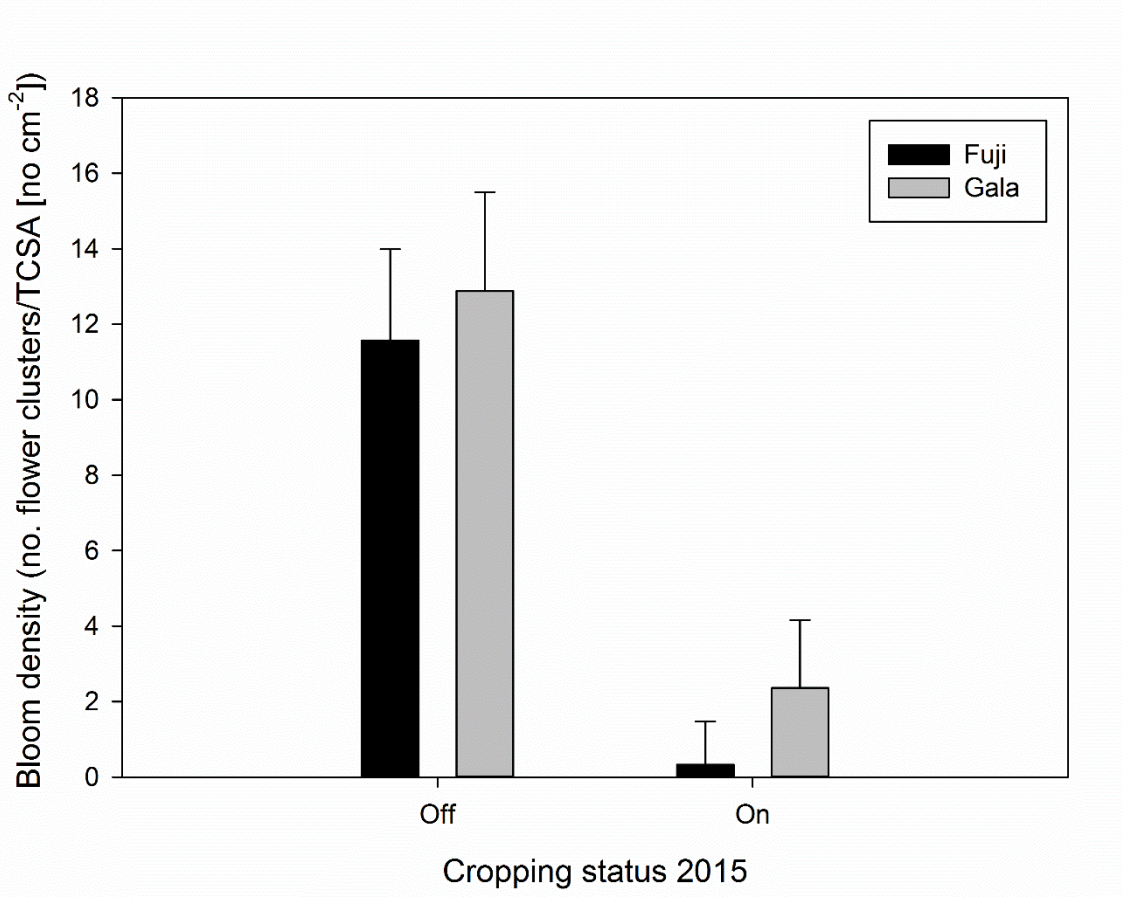

Figure 4 Bloom density expressed as mean (n = 65) number of flower clusters per trunk cross sectional area (cm²) in 2016 in response to the cropping status in 2015 for the cultivars ‘Fuji’ and ‘Gala’, respectively. Error bars indicate standard error.

In the 2015 growing season, the first initiated ‘Gala’ buds were found 99 dafb, irrespective of treatment, and bud initiation was observed until the last sampling date at 127 dafb (Fig. 5a). However, at each sampling time ‘off’ trees exhibited a much greater percentage of bud initiation than ‘on’ trees, resulting in average initiation percentages, calculated starting from the first sampling point where initiated buds were found until the last sampling date, of 87% and 33%

for ‘off’ and ‘on’ trees, respectively. Bud initiation in ‘Fuji’ started in ‘off’ trees at 77 dafb, 22 days earlier than in ‘Gala’, but at 120 dafb in ‘on’ trees, yielding a mean percentage of initiation of 83% for ‘off’ trees and 17% for ‘on’ trees (Fig. 5b).

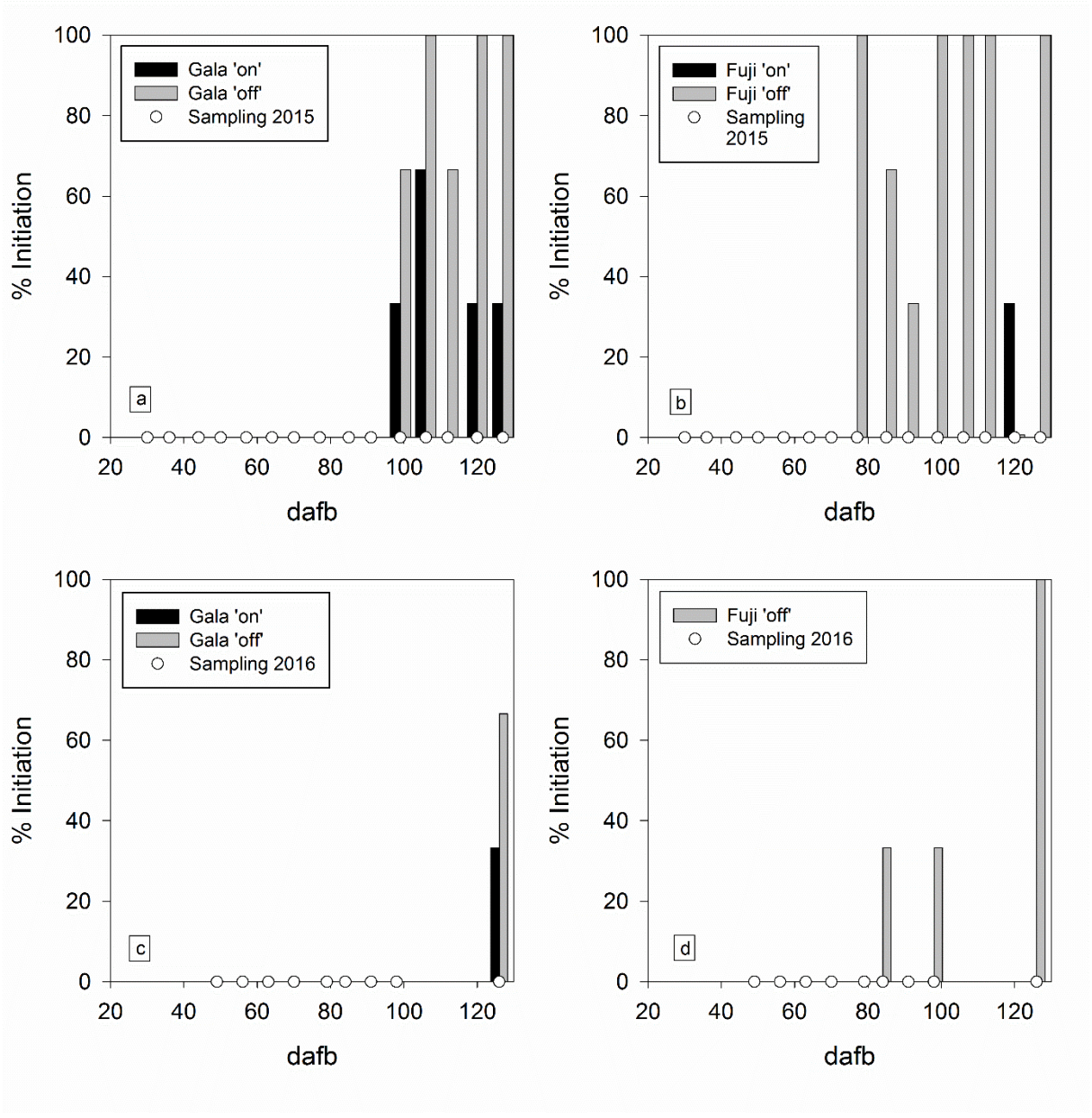

Figure 5 Mean percent bud initiation at days after full bloom, calculated separately at each sampling date, in ‘on’ and ‘off’ treatment, respectively, for the cultivars ‘Gala’ (a and c) and ‘Fuji’ (b and d) during two consecutive growing seasons. The ‘Fuji’ ‘on’ treatment is not shown since there was no initiated bud found in 2016.

The first initiated ‘Gala’ buds in 2016 were noted at 126 dafb, again irrespective of treatment (Fig. 5c). Since no buds were collected between 98 and 126 dafb, bud initiation may have started earlier and nearer to the onset of bud initiation in 2015. Nevertheless, similar to 2015 results, ‘Gala’ ‘off’ trees showed a 2-fold higher percentage of initiation (66%) than ‘on’ trees

(33%). In 2016, buds from ‘Fuji’ ‘off’ trees were first initiated at 84 dafb, which was close to the result in 2015; however, bud initiation was quite variable in subsequent samples. In contrast, buds from ‘Fuji’ ‘on’ trees were not initiated until the last sampling date (Fig. 5d).

The equations used to plot the probabilities of apple bud initiation as model outputs are given in Table 1.

Table 1 Equations for the probability of bud initiation and the initiation rate for the different cultivars, treatments and years. Due to the sampling gap in 2016, equations for ‘Gala’ were only established for the 2015 dataset. The ‘Fuji’ ‘on’ treatment was not included since there was no (2015) and one initiated bud (2016) noted. x = heat accumulation from full bloom (growing degree hours). The interaction terms ‘gdh’*‘treatment’ for ‘Gala’ (p = 0.09) and ‘gdh’*‘year’ for ‘Fuji’ (p = 0.51) were dropped from the model because their p-values were above 0.05.

	Probability of Initiation	Initiation Rate
Gala 2015 ‘off’	$y = \frac{\exp(-10.86 + 0.0004 * x)}{1 + \exp(-10.86 + 0.0004 * x)}$	$y = \left(0.1162 * \left(\frac{exp(-10.86+0.0004*x)}{1+exp(-10.86+0.0004*x)}\right)\right) * \left(1 - \left(\frac{exp(-10.86+0.0004*x)}{1+exp(-10.86+0.0004*x)}\right)\right)$
Gala 2015 ‘on’	$y = \frac{\exp(-13.32 + 0.0004 * x)}{1 + \exp(-13.32 + 0.0004 * x)}$	$y = \left(0.1162 * \left(\frac{exp(-13.32+0.0004*x)}{1+exp(-13.32+0.0004*x)}\right)\right) * \left(1 - \left(\frac{exp(-13.32+0.0004*x)}{1+exp(-13.32+0.0004*x)}\right)\right)$
Fuji ‘off’ 2015	$y = \frac{\exp(-8.00 + 0.0003 * x)}{1 + \exp(-8.00 + 0.0003 * x)}$	$y = \left(0.1104 * \left(\frac{exp(-8.00+0.0003*x)}{1+exp(-8.00+0.0003*x)}\right)\right) * \left(1 - \left(\frac{exp(-8.00+0.0003*x)}{1+exp(-8.00+0.0003*x)}\right)\right)$
Fuji ‘off’ 2016	$y = \frac{\exp(-9.57 + 0.0003 * x)}{1 + \exp(-9.57 + 0.0003 * x)}$	$y = \left(0.1104 * \left(\frac{exp(-9.57+0.0003*x)}{1+exp(-9.57+0.0003*x)}\right)\right) * \left(1 - \left(\frac{exp(-9.57+0.0003*x)}{1+exp(-9.57+0.0003*x)}\right)\right)$

The ‘Gala’ model could only be fitted to the data from 2015 due to sampling in 2016 not covering the bud initiation period. The main effects ‘gdh’ and ‘treatment’ were highly significant with p-values of 0.0002 and 0.0088, respectively. The interaction between ‘gdh’ and ‘treatment’ was removed from the model due to a non-significant effect in the Wald-χ^2-test (p = 0.09). The onset of bud initiation, defined as 20% of the maximum initiation rate, occurred at 21500 GDH (76 dafb) for ‘off’ trees and at 28122 GDH (96 dafb) for ‘on’ trees. Both treatments had the same slope for the probability of bud initiation (Fig. 6a).

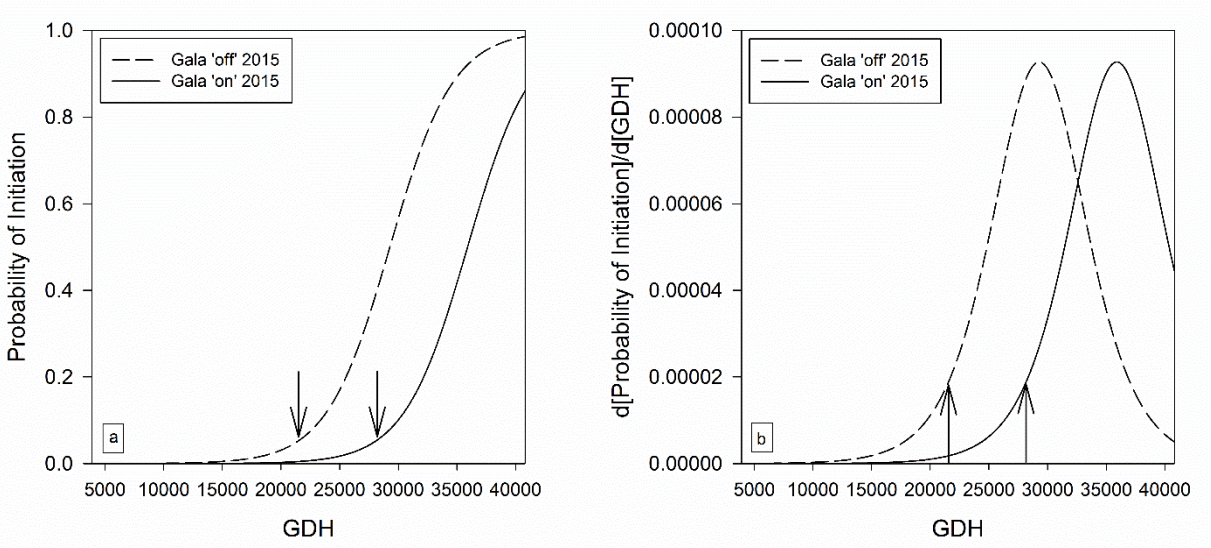

Figure 6 Modelled predictions of the probabilities of bud initiation (a) and the initiation rate (b) in 'Gala' 'on' and 'off' trees in 2015. Arrows indicate the onset of bud initiation defined as 20% of the maximum initiation rate: 21500 GDH (76 dafb) for 'Gala' 'off' trees in 2015; 28122 GDH (96 dafb) for 'Gala' 'on' trees in 2015.

Consequently, the maximum rate of bud initiation was also the same for both treatments (Fig. 6b) but it was reached 21 days earlier for 'off' (at 29282 GDH or 100 dafb) than for 'on' trees (at 35905 GDH or 121 dafb). The p-value for the Goodness-of-Fit (GOF) Test of Hosmer and Lemeshow (Hosmer and Lemeshow 2004) was 0.9, indicating a high goodness of fit for the model.

Since only one initiated bud was found for 'Fuji' 'on' trees, modelling this treatment was not possible. Thus, Figure 7 shows the predictions of the probabilities of bud initiation and the initiation rate in 'Fuji' 'off' trees for both consecutive growing seasons.

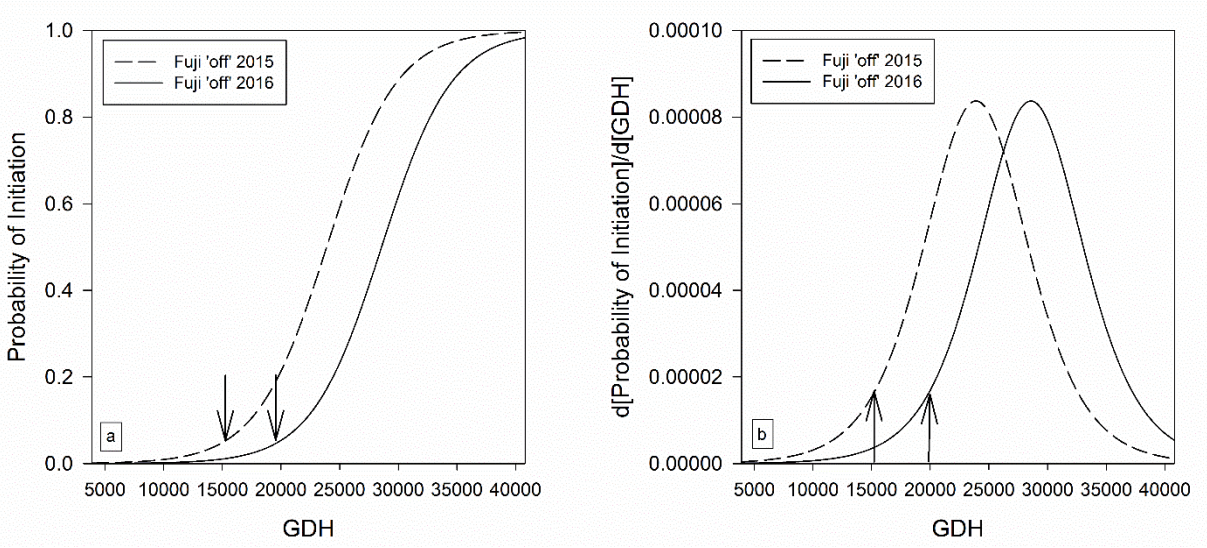

Figure 7 Modelled predictions of the probabilities of bud initiation (a) and the initiation rate (b) in 'Fuji' 'off' trees in 2015 and 2016. Arrows indicate the onset of bud initiation defined as 20% of the maximum initiation rate: 15274 GDH (57 dafb) for 'Fuji' 'off' trees in 2015; 19955 GDH (72 dafb) for 'Fuji' 'off' trees in 2016.

The main effect 'gdh' was highly significant with a p-value of 0.001, whereas the effect 'year' was not significant with a p-value of 0.0718. The interaction between 'gdh' and 'year' was dropped from the model since a non-significant effect was found in the Wald-χ^2-test ($p = 0.51$). The onset of bud initiation for 'Fuji' 'off' trees was 15274 GDH (57 dafb) in 2015 and 19955 GDH (72 dafb) in 2016. Both years had the same slope for the probability of bud initiation (Fig. 7a), resulting in an identical maximum rate of bud initiation that was reached 18 days earlier in 2015 (at 23893 GDH or 84 dafb) than in 2016 (at 28574 GDH or 102 dafb) (Fig. 7b). The p-value for the GOF Test of Hosmer and Lemeshow (Hosmer and Lemeshow 2004) was again not significant with 0.09, indicating a reasonable goodness of fit for the model. The length of the active initiation period, defined as the period with a rate of initiation of at least 20% of the maximum rate, was 49 days in 'Gala' 'off' trees in 2015, 63 days in 'Gala' 'on' trees in 2015, 53 days in 'Fuji' 'off' trees in 2015 and 2016, respectively. However, the maximum initiation rate was 5% lower in 'Fuji' 'off' trees compared to 'Gala' 'off' trees. A continued decline of the bud initiation rate to 20% was not observed in 'Gala' 'on' trees in 2015, because the sampling period ended shortly after the maximum rate was obtained.

The size of bud meristems was distinctly different between initiated and non-initiated buds (Fig. 8).

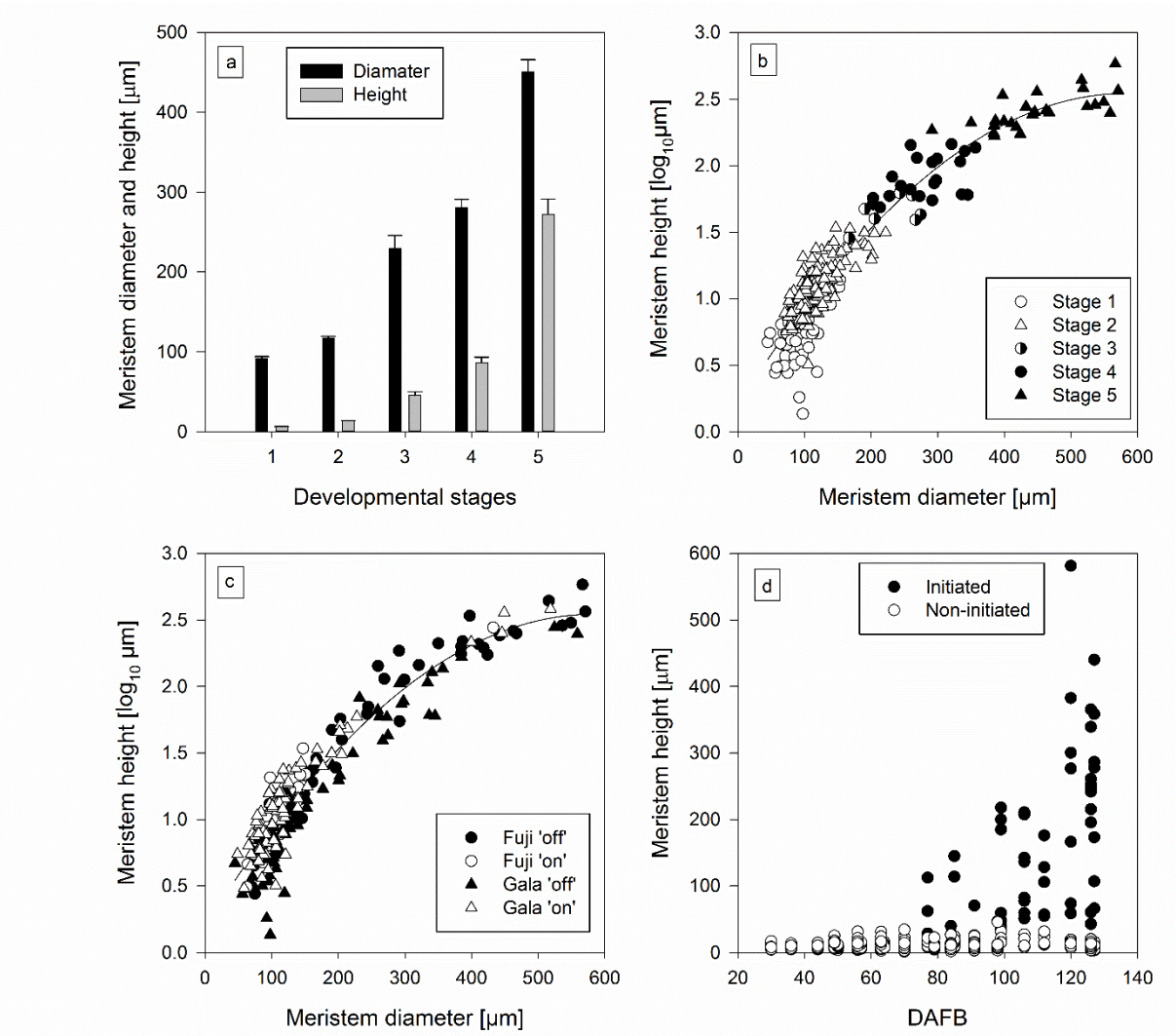

Figure 8 Mean height and diameter of bud meristems at each developmental stage across both cultivars and treatments (vertical bars are standard error) (a), relationship between meristem diameter and height at each developmental stage (b) as well as for each cultivar and treatment combination (c), and the time-dependent pattern of meristem height of initiated and non-initiated buds across both cultivars and treatments (d). All figures are based on data from 2015 and 2016. Equation for the regression line in (b) and (c): $y=-6.8723E\text{-}006x^2+0.008x+0.1975$ with $R^2=0.899$.

Stages 1 and 2, considered vegetative, did not vary considerably in height and diameter of the meristem (Fig. 8a), whereas the later stages, characterized by floral commitment, had larger meristems. Initiated buds had meristems with a diameter of at least 200 µm and a height of at least 32 µm (Fig. 8a, b). A regression analysis of all bud meristems in both experimental years suggested that meristem diameter is strongly correlated to meristem height (R^2 = 0.89), irrespective of the dataset sorted by meristem stage (Fig. 8b) or cultivar and crop load combination (Fig. 8c). The meristem height of initiated buds increased continuously from

77 dafb, the beginning of doming, until the last sample at 127 dafb, whereas it remained at constant level for non-initiated buds throughout that period (Fig. 8d).

2.4 Discussion

2.4.1 Meristem characteristics

Measuring the meristem size is a useful tool to describe the distinct morphological characteristics of initiated and non-initiated buds, which was also reported in a study assessing the development of buds in 'Starkspur Supreme Delicious' apple (Hirst and Ferree 1995). The beginning of doming at 77 dafb is in agreement with findings from other studies (Foster et al. 2003; Hoover et al. 2004), which observed pronounced doming in the majority of apple bud samples between 72 and 127 dafb. A bimodal distribution of vegetative meristem diameters as reported by others (Foster et al. 2003) was not observed in this study.

The non-observed initiation of buds in 'Fuji' 'off' trees at 91 dafb (Fig. 5d) indicates some degree of variability in floral bud development among sample dates and this biologically expected heterogeneity was also found in other studies (Pichler et al. 2007; Lauri et al. 2008).

2.4.2 Crop load

The model for the 'Gala' 2015 dataset shows, that the onset of floral bud initiation but not the rate of initiation was significantly affected by the 'on' and 'off' crop load treatments, respectively. Thus, under the specific climatic conditions in southwest Germany, trees bearing no fruit started bud initiation on 2-year-old spurs at 76 dafb, whereas this developmental process was delayed in heavy cropping trees until 96 dafb. This result is not in agreement with findings of other authors who reported that the period of flower bud induction begins sooner in 'on' than in 'off' trees (Li et al. 1995) or showed that the presence of fruit inhibited flower bud formation earlier on trees with heavy bloom than on those with light bloom (Marino and Greene 1981). However, the results show that environmental factors and tree cropping status may

explain the largely ambiguous and inconsistent published data for the onset of floral bud initiation in apple (McArtney et al. 2001).

Despite an 80% probability of bud initiation in ‘Gala’ ‘on’ trees at the end of the experimental period (Fig. 6a), return bloom in the following year was relatively small with just over 2 flower clusters cm^{-2} TCSA. We postulate that heavy cropping delays the onset of flower bud initiation to such extent that floral bud differentiation is poorly advanced prior to endodormancy, leading to little return bloom.

2.4.3 Cultivar

Although the experimental design did not allow for a statistical evaluation of cultivar differences, the data substantiate the commonly accepted notion that the genetic make-up of a cultivar profoundly affects the onset of flower bud initiation (Hoover et al. 2004). For example, the onset of bud initiation on ‘off’ trees was 57 dafb in ‘Fuji’ and 76 dafb in ‘Gala’ in 2015. A difference in floral initiation of approximately 19 days between ‘Fuji’ and ‘Gala’ was also found under the climatic conditions in New Zealand (Hoover et al. 2004); however, doming occurred much later in ‘Fuji’ (86 dafb) and ‘Gala’ (112 dafb). In another study in New Zealand (McArtney et al. 2001), the onset of floral bud initiation for ‘Royal Gala’ was between 72 to 99 dafb, which is similar to that of heavy cropping ‘Gala’ trees at 96 dafb in southwest Germany in our study. The percentages of bud initiation (Fig. 5) were studied for trees with an extremely high and no crop load, respectively. The treatment dependent differences found in this study may be reduced by thinning practices usually applied in commercial apple orchards. Indeed, appropriate thinning techniques can effectively regulate the percentage of bud initiation even in strongly biennial bearing cultivars (Wertheim and Webster 2005). However, in a different study (unpublished data) with three apple cultivars thinned to a medium crop load (approx. 4 fruit cm^{-2} TCSA), the percentage of initiated buds at 126 dafb was 68% for ‘Fuji’, 71% for ‘Gala’ and 98% for ‘Braeburn’. Although no significant differences were found, a clear cultivar-specific trend in floral bud initiation was evident. Moreover, the tree to tree variability in the percentage of initiated buds was much larger in ‘Fuji’ and ‘Gala’ than in ‘Braeburn’. These results are in

agreement with other findings (Krasniqi et al. 2013), where a heterogenous biennial bearing behaviour among trees of the same cultivar within the same orchard was found.

2.4.4 Heat accumulation

The onset of initiation for ‘Fuji’ ‘off’ trees occurred 5 days after summer solstice in 2015 (June 26) but 20 days after summer solstice in 2016 (July 11). The observation that flower bud initiation did not occur on the same day in both years supports the notion by other authors that flower bud initiation cannot be entirely and fully deterministically driven by daylength (Dolega and Link 2002; Heide and Prestrud 2005; Kurokura et al. 2013).

The difference in the onset of bud initiation for ‘Fuji’ ‘off’ trees between 2015 (15274 GDH or 57 dafb) and 2016 (19955 GDH or 72 dafb) was 23% for GDH or 21% for dafb (Fig. 7). GDH accumulated from full bloom explained better the existing annual variability in the onset of bud initiation than GDH from the transition from short- to long-day or GDH with a base temperature of 10°C as used in another study (McArtney et al. 2001). Moreover, the probabilities of bud initiation in both years nearly merge at approximately 40000 GDH, indicating some degree of recovery following the delayed onset of bud initiation in 2016. The results suggest that heat accumulation has a modulating effect on the pattern (onset, progression, termination) of bud initiation. There is some experimental evidence (Zhu et al. 1997), that flower bud initiation is advanced under higher temperatures; however, this was not supported by our results.

The difference in the onset of bud initiation in ‘Gala’ ‘off’ (21500 GDH or 76 dafb) and ‘Gala’ ‘on’ trees (28122 GDH or 96 dafb) in 2015 was clearly due to treatment effects and was 23.5% for GDH (Fig. 6) and 21% for dafb. If an earlier onset of flower bud initiation in heavy cropping trees would solely require a higher temperature demand, the onset in ‘Gala’ ‘on’ trees would occur at a similar time as ‘off’ trees when accumulating 23.6% more GDH 20 days earlier. However, it is more likely that a minimum temperature accumulation is required for triggering the onset of bud initiation, but a crop load mediated factor is delaying this process. This factor might be a decreased carbohydrate supply of the bud meristems in heavy cropping trees as already suggested in literature (Monselise and Goldschmidt 1982). Although the total

carbohydrate requirement for flower bud formation may be small compared with that of other sinks (e.g. fruit), there is now growing evidence that the proportion of carbohydrates available for flower bud formation might be limited due to a low priority rank order of buds and/or exhausted carbohydrate reserve pools. Excessive cropping in the 'on' year may reduce plant carbohydrate reserves and this deficiency may exert a carbohydrate-induced inhibitory effect of source limitation on flower bud induction.

2.5 Conclusion

The results from this study can be summarised in a proposed concept (Fig. 9), which identifies the main factors influencing the onset of flower bud initiation in apple.

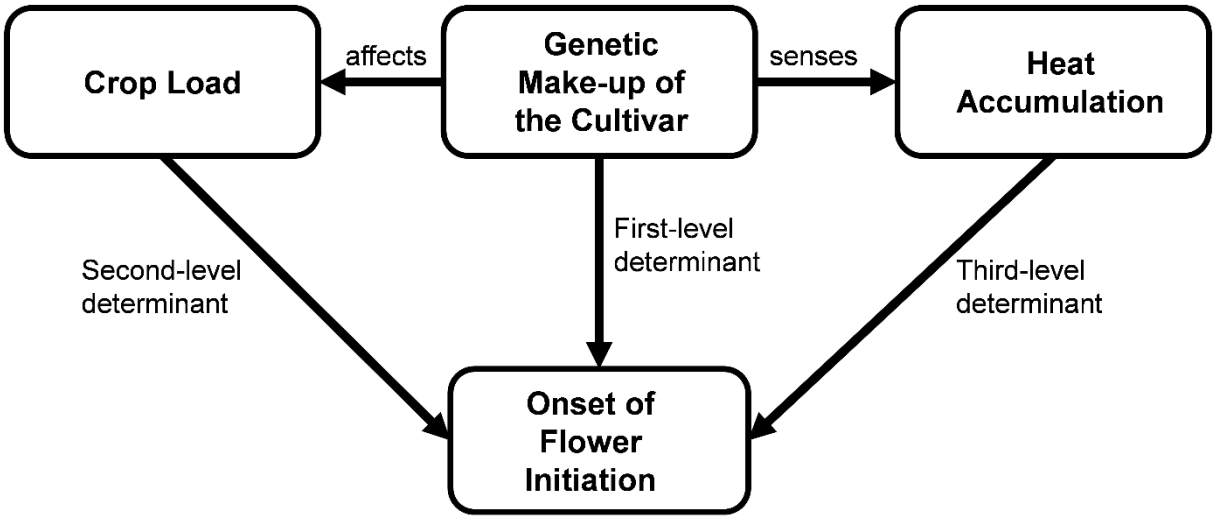

Figure 9 Proposed factors affecting the onset and duration of flower initiation in apple: the genetic make-up of the cultivar, crop load and heat accumulation.

In general, the genetic make-up of a given cultivar is the first-level determinant for the onset of flower bud initiation as indicated by the large differences between 'Fuji' 'off' and 'Gala' 'off' trees in 2015, despite being exposed to the same heat accumulation and crop load conditions. High crop load delayed considerably the onset of bud initiation in 'Gala', a response that may occur irrespective of cultivar. However, this response could not be modelled for the strongly biennial bearing cultivar 'Fuji' due to the absence of initiated buds in 'on' trees. It remains unclear whether this crop load driven (second-level determinant) response in the onset of

flower bud initiation is temperature-dependent. Nevertheless, the yearly differences in the onset of bud initiation are related to differences in heat accumulation (third-level determinant). To study the interactions between cultivar, crop load and temperature in relation to flower bud initiation requires specific experimental setups and can only be fully investigated in controlled-environment chambers.

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Author contribution statement

JK, AM, HF, MH and JW conceptualized and conducted the experiment; SS and NM prepared histological slides and provided parts of the data; FC assisted in the logistic regression modelling and statistics; JK wrote the first draft of the manuscript; AM wrote sections of the manuscript; JW, HF and MH critically revised the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

3. Proteomic Differences in Apple Spur Buds from High and Non-cropping Trees during Floral Initiation

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Julian Kofler[1], Anton Milyaev[1], Berit Wuertz[2], Jens Pfannstiel[2], Henryk Flachowsky[3], Jens-Norbert Wünsche[1]

[1]Institute of Crop Science, Section of Crop Physiology of Specialty Crops (340f), University of Hohenheim, Emil-Wolff-Straße 23, 70599 Stuttgart, Germany
[2]Mass Spectometry Unit, Core Facility Hohenheim (640), University of Hohenheim, August-von-Hartmann-Str. 3, 70599 Stuttgart
[3]Institute for Breeding Research on Fruit Crops, Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Pillnitzer Platz 3a, 01326 Dresden, Germany

Abstract
The cropping behavior of biennial apple (*Malus* ×*domestica* Borkh.) cultivars is irregular and often follows a biennial bearing pattern with 'On' years (high crop load and inhibited floral bud formation) followed by 'Off' years (little crop load and a promoted formation of floral buds). To study proteomic differences between floral and vegetative buds, trees of the strongly alternating cultivar 'Fuji' and the regular bearing cultivar 'Gala' were either completely thinned or not thinned at full bloom to establish two cropping treatments with no ('Off') or a high ('On') crop load, respectively. Student`s t-Tests indicated significant differences of protein profiles in buds from 2-year old spurs from both treatments at each sampling date. Abundance patterns of protein clusters coincided with the onset of floral bud initiation and were most noticeable in buds from 'On' trees with a decreased abundance of key enzymes of the phenylpropanoid and flavonoid pathways and an increased abundance of histone deacetylase and ferritins. Furthermore, an increased abundance of proteins involved in histone and DNA methylation was found in the buds from 'Off' trees. This study presents the first large-scale, label-free proteomic profiling of floral and vegetative apple buds during the period of floral bud initiation.

Keywords: alternate bearing, flower induction, *Malus* ×*domestica,* proteomic profiling

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3.1 Introduction

A crucial process in annual and perennial flowering plants is the development of floral meristems to enter the reproductive cycle and to ensure the formation of progenies. Proteins, steered by their respective transcripts or by interacting with other proteins, play an essential role in starting, conveying and executing floral development pathways. Prominent examples are the HEADING DATE 3a (Hd3a) protein acting as a mobile flowering signal in rice (Tamaki et al. 2007), the photoreceptor regulation of the CONSTANS (CO) protein in photoperiod-dependent flowering in *Arabidopsis thaliana* (Valverde et al. 2007), the control of flowering time in *Arabidopsis thaliana* through the spliceosome protein BAD RESPONSE TO REFRIGERATION 2 (BRR2) (Mahrez et al. 2016), the long-distance signaling of floral induction by FLOWERING LOCUS T (FT) protein movement in *Arabidopsis thaliana* (Corbesier et al. 2007) or the repression of floral development by FT protein`s close relative TERMINAL FLOWER 1 (TFL1) (Wigge 2011).

Apple (*Malus* ×*domestica* Borkh.) starts its 2-year reproductive cycle with the process of flower bud induction during early summer, when the vegetative meristems perceive an until now unknown signal that triggers floral bud development (Buban and Faust 1982; Hanke et al. 2007; Hättasch et al. 2008; Koutinas et al. 2010). Apple flowers are predominantly formed in the terminal buds of short shoots, so-called spurs (Fulford 1966; Koutinas et al. 2010). The first visible structural changes of the bud meristem appear during the second phase of floral bud development, termed floral bud initiation, when a pronounced doming and broadening of the bud apex appears (Foster et al. 2003; Hanke et al. 2007; Koutinas et al. 2010). Flower bud formation continues until autumn with the development of inflorescence primordia and floral organs. This third stage of flower bud differentiation is then temporarily interrupted with the onset of bud dormancy and completed in the following spring shortly before bud burst with the formation of pollen sacs and ovules (Tromp 2000; Wilkie et al. 2008).

A major constraint of obtaining constant and thus economically viable yields in commercial apple orchards is the irregularity of cropping due to biennial bearing (Jonkers 1979). It is characterized by large yields of small-sized fruit in ‘On’ years and low yields of over-sized fruit

in ‘Off’ years (Williams and Edgerton 1981). It is assumed that the competitive overlap of flower bud formation for the following season and fruit growth within the current season is the physiological reason for this cropping behavior (Pratt 1988; Dennis and Neilsen 1999; Koutinas et al. 2010). However, the specific causal mechanism of how a fruit inhibits or the absence of a fruit promotes the development of the subtending spur bud still remains unclear. Although several studies exist that address this topic at transcriptomic level (Li et al. 2018; Zuo et al. 2018; Li et al. 2019a) with changes in DNA and/or histone methylation pattern (Fan et al. 2018, 2019), data or coherent models are not available for explaining the difference between ‘On’ and ‘Off’ or biennial and regular bearing apple cultivars at proteomic level.

In this study, apple spur buds were collected during one growing season from trees with heavy and no crop load of two cultivars differing in their bearing behavior, respectively. The proteomic profile of spur buds at different developmental stages was analyzed and cultivar differences were identified. As part of a larger transdisciplinary and multifocal project identifying the underlying mechanisms of biennial bearing in apple, this study focuses on the time-dependent proteomic changes in floral and vegetative apple spur buds in an attempt to contribute to a holistic understanding of the induction of flower buds in apple.

3.2 Material and methods

3.2.1 Experimental design and bud sampling

The experiment was conducted at the Centre of Competence for Fruit Cultivation near Ravensburg in Southern Germany. Two apple cultivars with a different degree of biennial bearing behavior were used: ‘Fuji’, clone ‘Raku-Raku’, known to be a strongly biennial bearing cultivar, and ‘Gala’, clone ‘Galaxy’, known as a more regular bearer. The experimental plot consisted of 130 ‘Fuji’ and ‘Gala’ trees, respectively, each in two neighboring 65-tree rows next to one another. Half of the trees were completely flower thinned (‘Off’ trees) by hand at full bloom on 30 April 2015 (both cultivars), whereas the other half of the trees was not flower thinned and remained the high return bloom density (‘On’ trees). Setting up these extreme crop

load treatments was done (i) to synchronize bud development within the trees to obtain homogenous bud samples from each trees, (ii) to increase flower bud induction in ‘Off’ trees and (iii) to inhibit flower bud induction in ‘On’ trees. Sampling started in the fourth week after full bloom (afb) and continued weekly for 15 weeks until 2 September 2015. At each sampling date, three randomly selected trees (one tree representing one biological replicate) per cultivar and treatment were selected and 55 buds from 2-year-old spurs were randomly sampled off each tree. During the sampling period, no tree was selected more than once. An overview of the experimental design and sampling procedure is given in Figure 1.

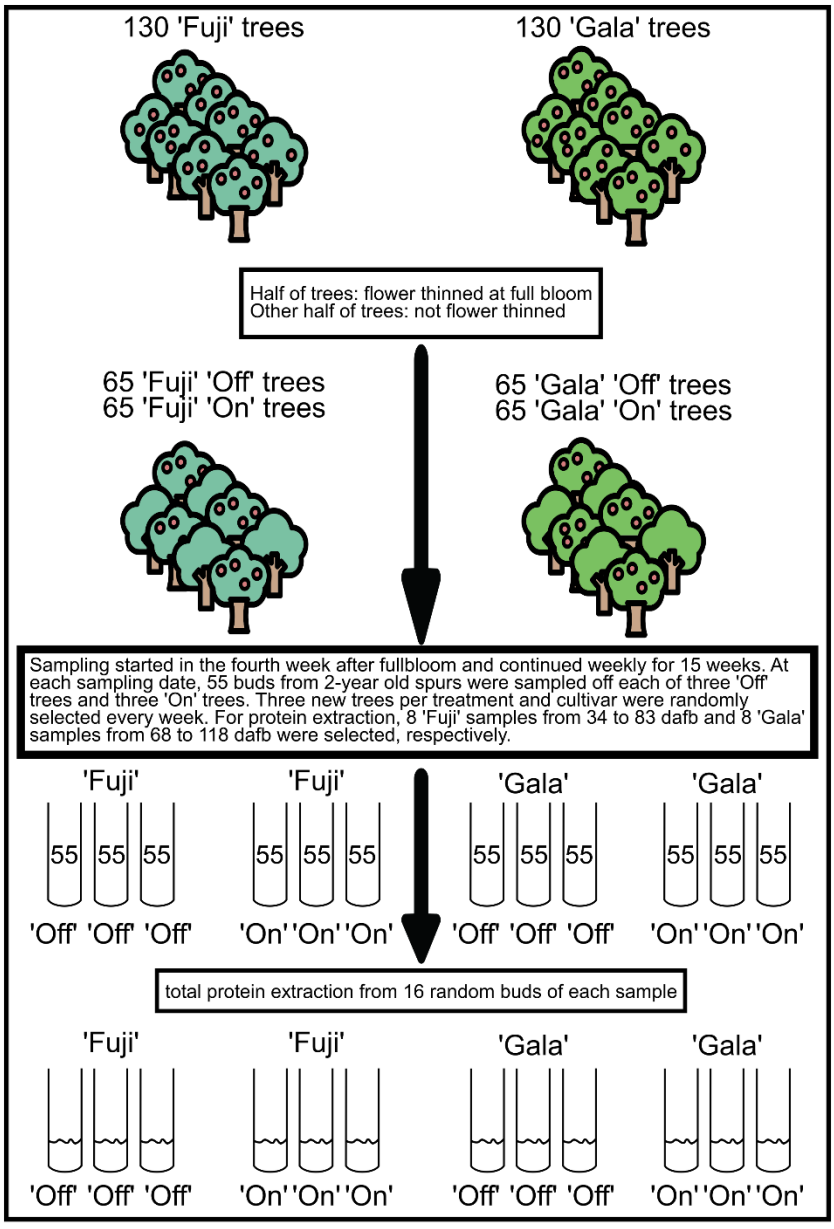

Figure 1 Schematic workflow figure of experimental design, bud sampling and protein extraction.

Immediately thereafter, buds with their scales removed were snap-frozen in liquid nitrogen and stored in safe-lock tubes at -80°C until further processing. The developmental stages of the buds were determined using histological analysis as described in detail (Kofler et al. 2019). From those results, eight continuous sampling dates were selected to cover the periods of flower bud induction and initiation in both cultivars, respectively, as shown in Figure 2. Due to the different bud initiation starting point (Figure 2), different sampling dates were selected for 'Fuji' and 'Gala'. For 'Fuji', samples from 34 to 83 days after full bloom (dafb) and for 'Gala' samples from 68 to 118 dafb were selected, respectively. This approach permitted cultivar comparison since floral bud development in 'Gala' started later than in 'Fuji' and in 'Gala' also crop load comparison (Milyaev et al. 2018).

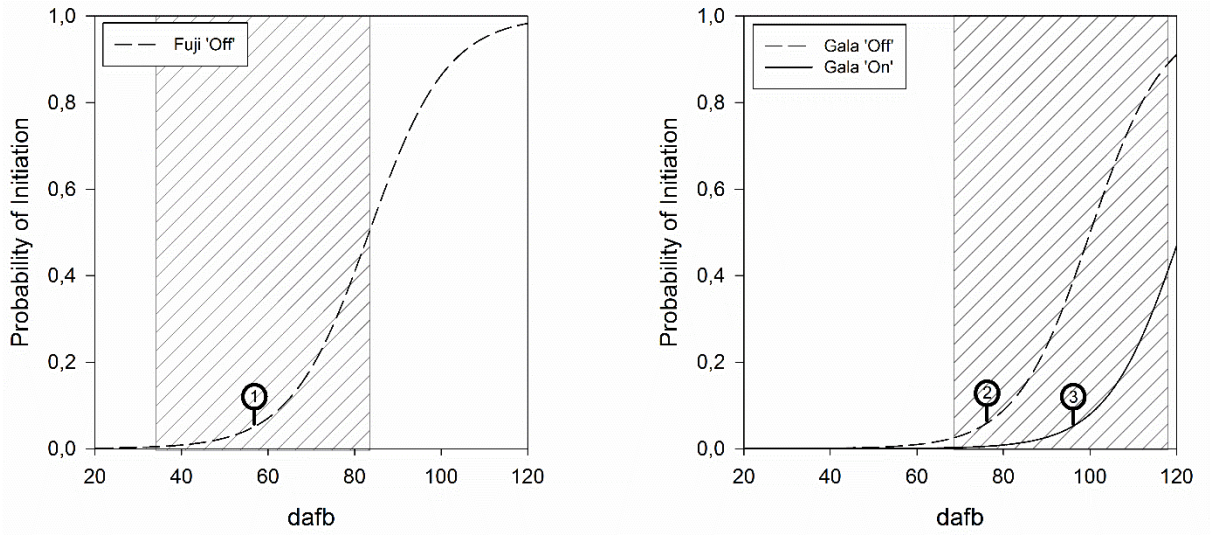

Figure 2 Calculated prediction of flower bud initiation based on histological data (Kofler et al. 2019). Starting point of bud initiation for 'Fuji' 'Off' was 57 dafb (1), for 'Gala' 'Off' 76 dafb (2) and for 'Gala' 'On' 96 dafb (2), respectively. No starting point of bud initiation could be calculated for 'Fuji' 'On' due to a lack of initiated buds. Shaded area indicates the time window for proteomic profiling: 34 dafb to 83 dafb for 'Fuji' and 68 dafb to 118 dafb for 'Gala'.

3.2.2 Protein extraction

Sixteen buds per tree were pooled together constituting one biological replicate (600 mg fresh weight). The sixteen randomly selected buds were ground in a cryogenic mixer mill (CryoMill, Retsch GmbH, Haan, Germany) cooled with liquid nitrogen. To achieve a homogenous pulverization, four buds were put in one safe-lock tube with two stainless steel balls of 5 mm diameter and four safe-lock tubes were ground in two grinding cycles with each 3 min long at the frequency of 25 Hz. Immediately after grinding, 1.5 ml of freshly prepared lysis buffer

(150 mM Tris-HCl, 2% sodium dodecyl sulfate, 20 mM dithiothreitol, adjusted to pH 6.8) was added to each safe-lock tube (Afzal et al. 2020). After shaking and 3 min incubation at room temperature, the milling balls were removed using a magnet and the samples were centrifuged for 8 min at 20,000 rcf. All four supernatants from one replicate were transferred into a single 15 ml conical centrifuge tube. An aliquot of 200 µl was transferred to a new 1.5 ml safe-lock tube (Eppendorf AG, Hamburg, Germany) for protein precipitation using chloroform/methanol (Afzal et al. 2020) and the obtained protein pellet was stored in digestion buffer (6 M urea, 50 mM Tris-HCl pH 8.5) at 4°C until further processing. Protein concentration was determined using the Bradford assay (Kielkopf et al. 2020) and 25 µg of proteins were digested in-solution using a mixture of Trypsin (Roche Pharma AG, Basel, Switzerland) and Lys-C (Wako Chemical GmbH, Neuss, Germany) (Saveliev et al. 2013). Peptides were desalted and purified using the C18-StageTips method (Rappsilber et al. 2003) and resuspended in 20 µl of 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, USA). Four quality control samples were created, each representing a pooled sample of 24 out of 96 randomly drawn samples, resulting in 100 samples in total.

3.2.3 ESI-MS method

Nanoscale liquid chromatography electrospray ionization tandem mass spectrometry (Nano-LC-ESI-MS/MS) analysis was performed on an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, USA) coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, USA), using an EASY-Spray nanoelectrospray ion source (Thermo Fisher Scientific, Waltham, USA), at the Core Facility Hohenheim, Mass Spectrometry Unit (University of Hohenheim, Stuttgart, Germany). Tryptic peptides were injected directly to an EASY-Spray analytical column (PepMap RSLC C18, 2 µm 100 Å 75 µm x 250 mm column, Thermo Fisher Scientific, Waltham, USA), operated at a constant temperature of 40°C. Gradient elution was performed at a flow rate of 250 nl min^{-1} using two solvents. 0.5% acetic acid (solvent A) and 0.5% acetic acid in 80% acetonitrile (solvent B) during 140 min with the following gradient profile: equilibration with 10 µl of 3% solvent B, followed by a gradient of 3% - 10% of solvent

B in 50 min, 10% - 22% solvent B in 40 min, 22% - 45% solvent B in 25 min, 45% - 95% solvent B in 10 min, 15 min isocratic at 95 % solvent B. The mass spectrometer was operated using Xcalibur software (version 4.0.27, Thermo Fisher Scientific, Waltham, USA). Survey spectra (m/z = 300-1600) were detected in the Orbitrap at a resolution of 70,000 at m/z = 200. Data dependent tandem mass spectra were generated for the 10 most abundant peptide precursors in the Orbitrap. For all Orbitrap measurements, internal calibration was performed using lock-mass ions from ambient air (Olsen et al. 2005).

3.2.4 Protein identification and statistics

MS data were analyzed using MaxQuant (version 1.6.2.10) (Cox and Mann 2008) in the label-free quantification (LFQ) mode with carbamidomethylation of cysteine residues as fixed modification and oxidation of methionine and n-terminal acetylation as variable modifications. A maximum of three missed cleavages was allowed and the match between runs function was switched on. The peak list was searched against all 45116 FASTA-formatted protein sequences from the GDDH13 apple genome version 1.1 (Daccord et al. 2017).

LFQ intensities calculated by MaxQuant were loaded into Perseus (version 1.6.10) (Tyanova et al. 2016) for statistical analysis. Reverse hits, identifications only by site and potential contaminants were removed and LFQ intensities log2(x) transformed. The unique peptide threshold was set to the minimum of two and the valid values filter was set to a minimum of three valid values during at least one sampling date in at least one cultivar and treatment. Missing values were imputed with random numbers from the normal distribution and a down shift of 1.8 only to create the partial least-squares discriminant analysis (PLS-DA) which requires all data points to be present. PLS-DA was computed using MetaboAnalyst 5.0 (Pang et al. 2021). A two-sided Student`s t-Test between the treatments was performed separately for the cultivars (‘Fuji’ ‘Off’ vs. ‘Fuji’ ‘On’ and ‘Gala’ ‘Off’ vs. ‘Gala’ ‘On’) at each sampling date with 250 randomizations, the Benjamini and Hochberg false discovery rate (FDR) (Diz et al. 2011) of 0.01 and the bio-weight factor (Rosenfeld et al. 2004) as significance criteria by setting

the S0 value to 0.01. No missing values were imputed for the Student`s t-Tests. The number of degrees of freedom for the two samples Student`s t-Tests was

$(n_1+n_2)-2 = 4$ with $n_1=3$ and $n_2=3$. Only proteins that were significantly different between treatments during at least one sampling date were considered for further analysis. Intensities were then z-score normalized, where the mean of each row (protein) is subtracted from each value from that row (protein) and the result is divided by the standard deviation of the row. Hierarchical clustering was performed with Euclidean distance, linkage type average, preprocessing with k-means and a fixed cluster number of four. The mean value and standard error of all z-scored protein abundances belonging to one cluster was calculated at each date and plotted against dafb using SigmaPlot 12.3 (Systat Software Inc., San Jose, USA).

Protein sequences from the resulting eight clusters were blasted against the non-redundant NCBI protein sequence database with the specific taxonomy 3750 (*Malus ×domestica*) with word size 3 and a High Scoring Pairs (HSP) length cutoff of 33 using OmicsBox 1.2.4 (BioBam Bioinformatics S.L., Valencia, Spain) for annotation with descriptions, Gene Ontology terms (GO), InterPro GO terms and enzyme codes from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al. 1999) pathway database. Functional protein interaction analysis was done using STRING version 11.0 (Szklarczyk et al. 2019) by uploading multiple fasta-formatted amino acid sequences. As a reference organism, we chose *Arabidopsis thaliana* because preliminary functional analysis revealed that reference organisms such as *Malus ×domestica* or *Fragaria vesca* yielded less identified functional clusters and characterizations. Using the protein sequences from all eight clusters, the reference organisms *Malus ×domestica* and *Fragaria vesca* resulted only in 42 and 41 functional clusters, respectively, whereas *Arabidopsis thaliana* resulted in 52 functional clusters. Functional annotation clustering was done using the MCL algorithm with an inflation parameter of three (Brohée and van Helden 2006).

3.3 Results and discussion

In total, 7,194 different proteins were identified in our proteomics experiment. The average count of peptides per sample prior to all filtering steps was 41,699, the average count of unique peptides was 17,115 and the average count of identified proteins was 3,970. 6,025 proteins corresponding to 13.4% of the total apple proteome (Daccord et al. 2017) remained after removing all proteins with less than two unique peptides. Subsequently, all proteins were discarded that did not fulfill the criteria of having three LFQ values in at least one cultivar-date-treatment combination. 4,020 proteins remained for further analysis corresponding to 8.9% of the total apple proteome (Annex 3).

As shown in Figure 3, the PLS-DA shows variation between samples with two main components explaining 17.1% of the variance. The quality control samples cluster next to each other, indicating high reproducibility of sample preparation and subsequent Nano-LC-MS/MS analysis. While a basic separation between cultivars could be observed, the PLS-DA was not able to differentiate clearly between treatments. This might be explained by two factors that cause variability among bud samples. First, bud development underlies a certain within-tree heterogeneity, which leads to the circumstance that a random sample of collected buds from one tree at a certain time always includes buds that are differentially progressed in their development (Lauri et al. 2008). Second, a certain biologically expected variability is also observed between trees of the same cultivar in the same field (Pichler et al. 2007).

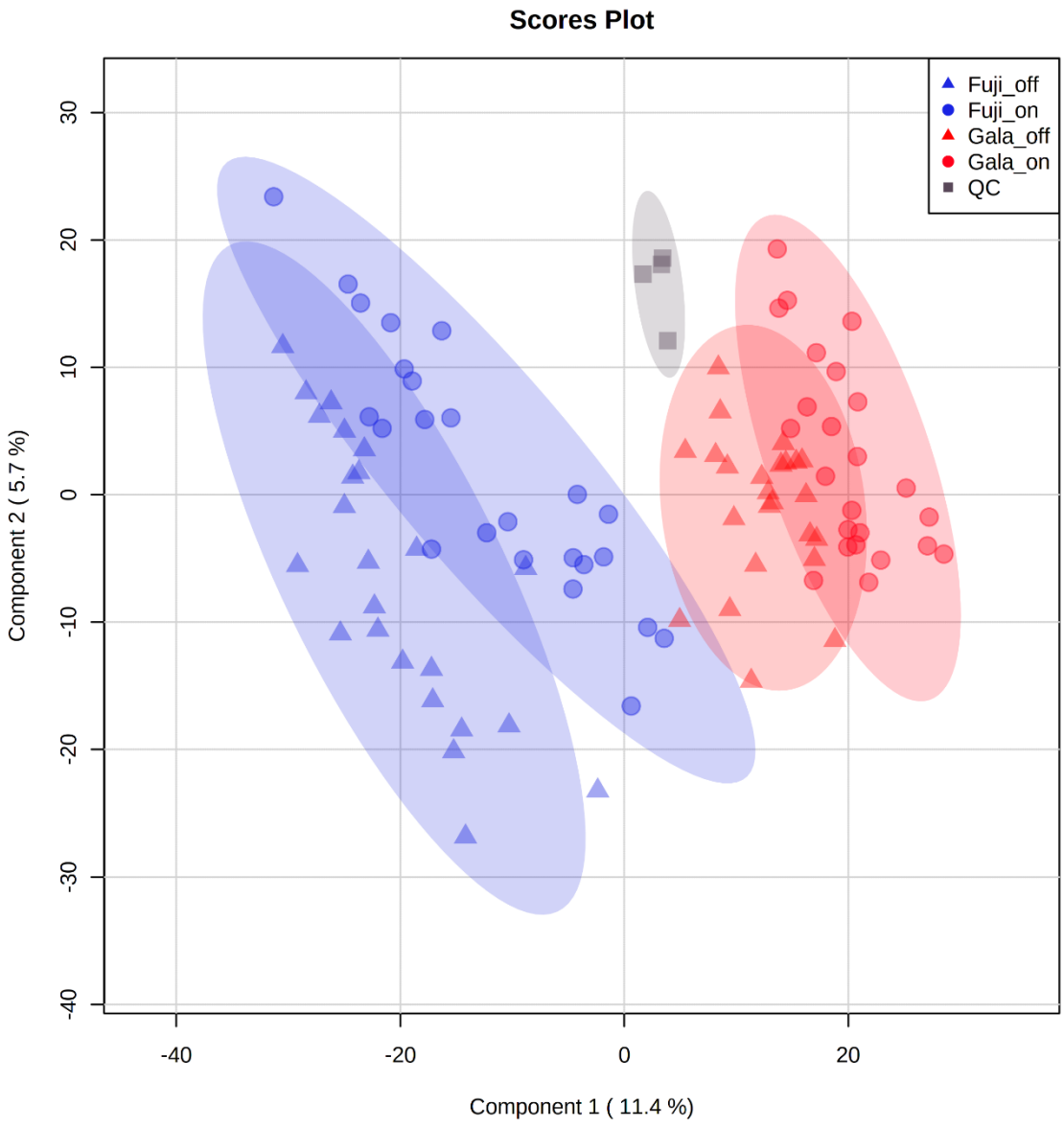

Figure 3 PLS-DA of all samples including quality control samples (QC). Missing values were imputed with random numbers from the normal distribution. Blue: 'Fuji'; red: 'Gala'; grey: QC; triangular: 'Off'; circle: 'On'; square: QC. Filled ellipses: 95% confidence region.

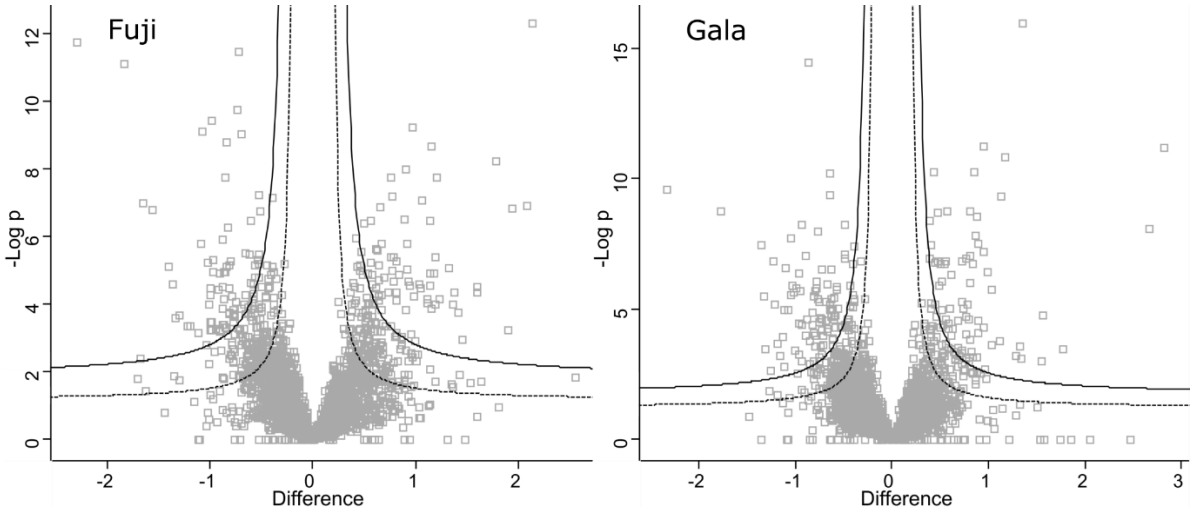

Figure 4 Volcano plots showing the difference in log2FC of protein abundance in buds from 'On' and 'Off' trees for the cultivars 'Fuji' and 'Gala'. Each square represents the relative abundance of one protein species. Positive differences refer to a higher abundance in buds from 'On' trees, negative differences refer to higher abundance in buds from 'Off' trees. Solid line represents class A significance criteria with FDR=0.01 while the dashed line represents class B significance criteria with FDR=0.05.

The results of global Student`s t-Tests between treatments within each cultivar are shown in Figure 4. A marked difference of the abundance of proteins between the two treatments shows that two different developmental pathways have been triggered.

3.3.1 Cultivar 'Fuji'

The Student`s t-Tests performed at each sampling date resulted in 159 proteins that were significantly different in abundance between samples from 'Fuji' 'On' and 'Off' trees during at least one sampling date while 17 out of 159 were significantly different at two sampling dates. 93 were higher abundant in buds from 'On' trees and 66 were higher abundant in buds from 'Off' trees. Overall, significant treatment differences in abundance of protein species existed with one protein at 34 dafb, 23 proteins at 62 dafb, 31 proteins at 68 dafb and 121 proteins at 75 dafb. The z-scores of the following clusters represent the mean z-scores of all protein abundances belonging to the various clusters. Clusters 46, 90 and 155 consist of proteins that are more abundant in buds from 'On' trees, whereas cluster 132 consists of proteins that are more abundant in buds from 'Off' trees. Cluster analysis presents four clusters of protein species, showing distinct abundance profiles over time (Figure 5).

Cluster 132, containing 43 proteins, was more abundant in buds from 'Off' trees than those from 'On' trees throughout the entire experimental period with a mean log2FC of -1.4 and a

pronounced treatment separation starting shortly after the onset of bud initiation (Figure 5). The decrease in buds from ‘On’ trees, which were mostly vegetative buds, during the onset of bud initiation might indicate that this cluster consists of proteins that advance floral bud development. MADS-box proteins and other transcription factors related to flowering could not be identified with the exemption of a K-box region and MADS-box transcription factor family protein (MD09G1009100). However, the protein was not identified in enough samples to perform a statistical analysis. Similar difficulties were observed also in a proteomics study on transcription factors involved in the regulation of flowering in *Adonis amurensis* (Zhou et al. 2019). The protein with the highest log2FC in cluster 132 was a caffeoyl-CoA 3-O-methyltransferase (MD13G1117900, EC:2.1.1.267, F:GO:0008171) that significantly differed at 68 and 75 dafb with a log2FC of -2.47. The protein is part of the flavone and flavonol biosynthesis pathway (ec00944) where it catalyzes the synthesis of the flavonols Laricitrin and Syringetin (Ogata et al. 1999) and was also found to be involved in the initiation of bud dormancy-release in *Pinus sylvestris* (Bi et al. 2011). STRING analysis of cluster 132 shows six functional clusters. The largest functional cluster with six proteins includes two S-adenosylmethionine synthetases (SAMS), SAM1 and SAM2, that catalyze S-adenosylmethionine. In rice, SAMS is essential for histone and DNA methylation to regulate gene expression related to flowering as the knockdown of SAMS genes resulted in late flowering phenotypes (Li et al. 2011). The phenylalanine ammonia-lyase (PAL1), the key enzyme of the phenylpropanoid pathway (Wanner et al. 1995), was at the center of a cluster belonging to the phenylpropanoid and flavonoid biosynthetic process. PAL1 was accompanied by Naringenin,2-oxoglutarate 3-dioxygenase (F3H), an intermediate in the biosynthesis of flavonols, which is possibly related to the synthesis of Naringenin and was shown to accumulate during the onset of dormancy in peach buds (Erez and Lavee 1969). Part of the cluster is also LDOX, a leucoanthocyanidin dioxygenase associated to the phenylpropanoid pathway synthesizing anthocyanin (Abrahams et al. 2003) and a putative caffeoyl-CoA O-methyltransferase (CCOAMT).

Cluster 155 contains 24 proteins with an average log2FC of -0.31; however, there was no treatment related difference in abundance during the period of bud initiation (Figure 5). The protein with the highest log2FC in cluster 155 was calmodulin (MD14G1241000, P:calcium-mediated signaling; F:calcium ion binding) that was significantly different at 75 dafb with a log2FC of -3.17. Calmodulin is the predominant calcium receptor in eukaryotes and essential for the Ca^{2+}/calmodulin-mediated signal network in plants (Yang and Poovaiah 2003). STRING analysis of cluster 155 resulted in three functional clusters. However, no relevant functions related to bud development could be attributed to the proteins of cluster 155, as expected by the similar abundances of the protein clusters in 'On' and 'Off' buds.

Cluster 46, containing 47 proteins with an average log2FC of 1.4 was more abundant in buds from 'On' trees than those from 'Off' trees with a significant difference at 75 dafb (Figure 5). Consequently, proteins belonging to cluster 46 could be involved in vegetative growth. The protein with the highest log2FC in cluster 46 is a plasma membrane intrinsic protein, probably aquaporin PIP2 (MD07G1174700, P:transmembrane transport; F:channel activity; C:membrane), with a log2FC of 2.49. STRING analysis of cluster 46 shows seven functional clusters mainly associated with responses to abiotic stimuli such as light and temperature and immune responses to pathogens. Six proteins that were more abundant in buds from 'On' trees than those from 'Off' trees at 75 dafb belong to the photosystem one and photosystem two oxygen evolving complex (LHCA3, LHCA4, LHCA5, CAB1, DRT112 and AT4G01150) located in the thylakoids.

Cluster 90 contains 45 proteins with an average log2FC of 1.5 and showed an increasing abundance in 'Fuji' 'On' buds throughout the experimental period with the highest number of significant hits (38) at 75 dafb (Figure 5). There was a clear difference between buds from 'On' and 'Off' trees, starting to become pronounced at 48 dafb, 9 days prior to the calculated onset of bud initiation at 62 dafb (Kofler et al. 2019). The protein with the highest log2FC in cluster 90 was an uncharacterized protein (MD09G1119300) with log2FC of 2.64. It shows sequence similarity to the fruit protein pKIWI501 from *Pyrus bretschneideri* (mean similarity 75.25%) and also to the cytochrome c1 from *Prunus dulcis* (Similarity 56.3%). STRING analysis of cluster 90

shows five functional clusters. At the center of a cluster with seven proteins is ferretin-1 (FER1). Loss of ferritins resulted in a reduced growth and strong defects in flower development in *Arabidopsis thaliana* (Ravet et al. 2009). Hence, the increase of ferritin-1 in ‘Fuji’ ‘On’ buds possibly promotes growth of vegetative buds. Another cluster with seven proteins consists of granule-bound starch synthase 1 (GBSS1) that is required for the synthesis of amylose and alpha-galactosidase 2 (AGAL2), which has an essential function during leaf development in *Arabidopsis thaliana* (Chrost et al. 2007) indicating a possible involvement in vegetative growth. This is supported by the increase of abundance of cluster 90 in buds from ‘On’ trees. There is also a cluster with two proteins belonging to the small interfering RNA (siRNA) biogenesis: AT2G37020.2, a translin family protein, and AGO4, an argonaute family protein that directs chromatin modifications, including histone methylation and DNA methylation (Zilberman et al. 2003).

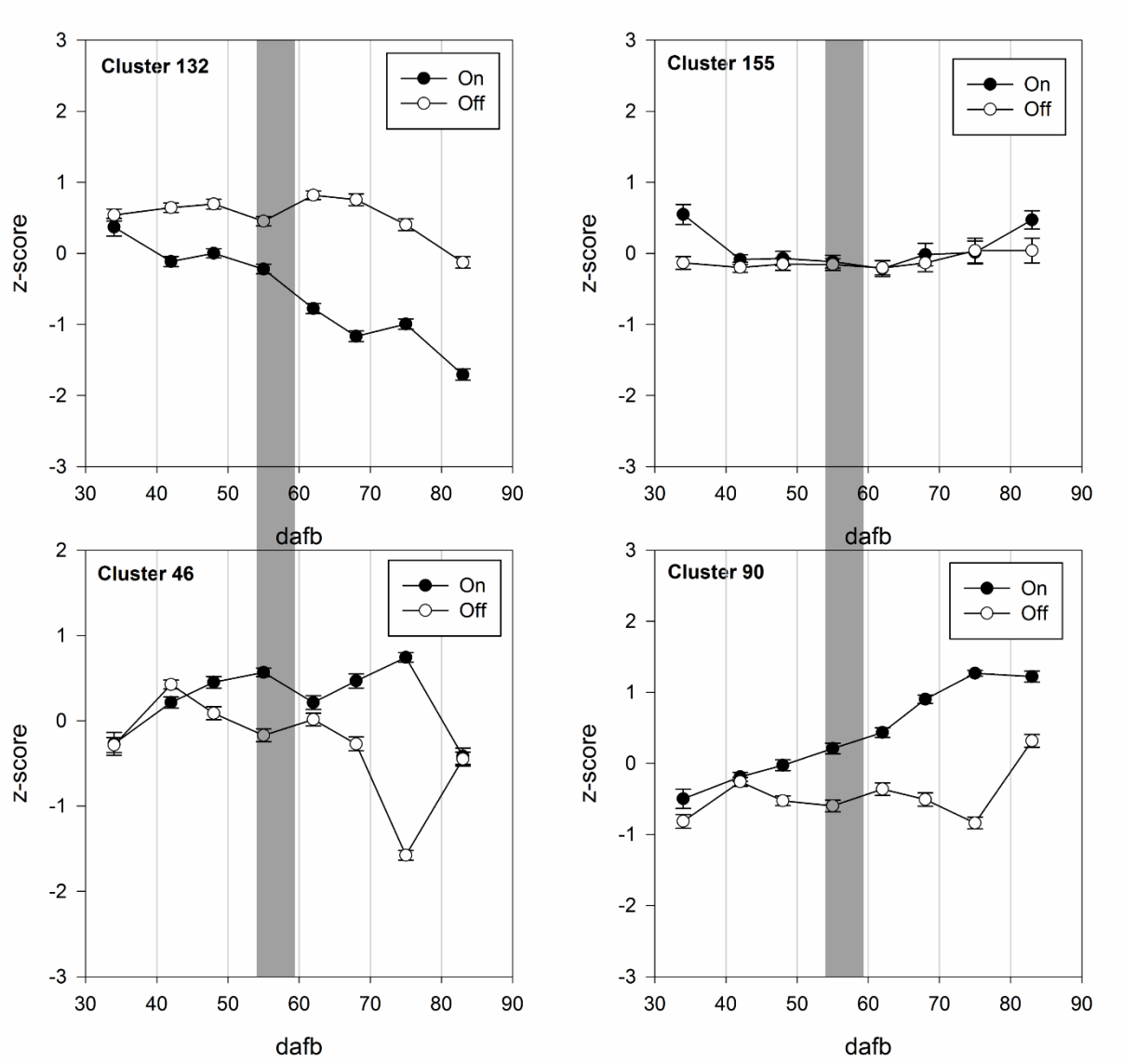

Figure 5 Normalized means of relative protein abundances from each cluster in buds from 'Fuji' 'On' and 'Off' trees. All proteins that were used for hierarchical clustering showed a significant difference between 'On' and 'Off' during at least one time point at FDR=0.01. Error bars indicate standard error of z-score. Grey bar indicates onset of bud initiation for 'Fuji' 'Off' at 57 dafb as identified by histological analysis (Kofler et al. 2019). Cluster 132: n=43; cluster 155: n=24; cluster 46: n=47; cluster 90: n=45.

Table 1 shows the top 20 proteins that have the highest (more abundant in buds from 'On' trees) and lowest (more abundant in buds from 'Off' trees) log2FC for the cultivar 'Fuji', respectively.

Table 1 The 40 key proteins with the highest log2FC between bud samples from 'Fuji' 'On' and 'Off' trees with corresponding GDDH13 ID, description, gene symbol, indication at which sampling date the difference was significant at FDR=0.01, p-value, log2FC 'On' over 'Off', count of unique peptides per protein, cluster affiliation and biological process. 40 shown out of 159 (Annex 1).

ID	Description	Gene symbol	dafb*	-log(p)	log2FC On-Off	Unique peptides	Cluster	GO Biological process
MD09G111930	fruit protein pKIWI501	LOC10341128	62, 68	2.64	3.67	14	90	
MD09G126760	embryonic protein DC-8-like	LOC10344421	62, 68	3.47	3.14	21	90	
MD08G106740	aminopeptidase M1-like	LOC10344078	75	2.55	3.13	12	90	P:proteolysis
MD06G112210	granule-bound starch synthase 1,	LOC10343745	75	2.36	2.72	10	90	
MD12G117850	ferritin-4, chloroplastic-like	LOC10343042	68, 75	2.74	2.56	9	90	P:iron ion transport; P:cellular iron ion
MD07G117470	plasma membrane intrinsic protein subtype 2	LOC10342869	68, 75	2.59	2.49	4	46	P:transmembrane transport
MD09G108140	probable endo-1,3(4)-beta-glucanase	LOC10342595	62, 75	2.53	2.27	13	90	
MD05G131910	polyphenol oxidase	LOC10340884	75	3.63	2.27	6	90	P:pigment biosynthetic process; P:oxidation-
MD11G113340	beta-galactosidase 8	LOC10343000	75	2.71	2.17	7	90	P:carbohydrate metabolic process
MD08G124410	elongation factor 1-gamma	LOC10344203	75	2.80	2.10	12	46	P:translational elongation
MD06G119500	chlorophyll a-b binding protein P4,	LOC10343782	75	2.20	2.10	2	46	P:photosynthesis, light harvesting
MD06G116940	small nuclear ribonucleoprotein-associated	LOC10341955	75	2.87	2.08	6	46	P:mRNA splicing, via spliceosome
MD00G106010	pre-mRNA-processing factor 39 isoform X1	LOC10343575	75	2.28	2.05	15	46	
MD15G122350	sucrose synthase	LOC10340090	75	2.27	2.05	18	46	P:sucrose metabolic process
MD09G105470	uncharacterized protein At5g39570-like	LOC10341699	75	2.23	1.99	20	46	
MD01G119900	3-hexulose-6-phosphate isomerase	LOC10344055	75	2.23	1.95	5	90	
MD09G101130	sulfite oxidase	---NA---	75	3.24	1.93	9	90	P:oxidation-reduction process
MD15G104400	aspartyl protease family protein At5g10770-	LOC10344242	75	2.37	1.93	13	46	P:proteolysis
MD06G100870	catalase isozyme 1	LOC10341210	75	2.45	1.93	37	90	P:response to oxidative stress; P:oxidation-
MD14G118860	protein EDS1L-like	LOC10341524	75	2.15	1.92	44	46	P:lipid metabolic process
MD10G100970	ATP phosphoribosyltransferase 1,	LOC10342943	62	3.39	-1.58	7	132	P:histidine biosynthetic process
MD11G114330	UDP-glucuronic acid decarboxylase 6 isoform	LOC10341298	62	3.01	-1.60	14	132	P:D-xylose metabolic process
MD15G137260	phospho-2-dehydro-3-deoxyheptonate	LOC10341947	68	3.60	-1.62	5	132	P:aromatic amino acid family biosynthetic
MD17G128340	S-adenosylmethionine synthase 1	LOC10340596	62	2.96	-1.64	8	132	P:S-adenosylmethionine biosynthetic process
MD11G124170	protochlorophyllide reductase, chloroplastic	LOC10340801	68	3.12	-1.65	23	132	P:oxidation-reduction process
MD07G122830	biotin carboxyl carrier protein of acetyl-CoA	LOC10343287	62	2.85	-1.68	5	155	
MD14G101080	NADH--cytochrome b5 reductase 1	LOC10343092	68	2.84	-1.75	5	132	P:oxidation-reduction process
MD05G116820	chloroplast envelope quinone oxidoreductase	LOC10343588	62, 68	3.35	-1.86	3	132	P:oxidation-reduction process
MD00G104090	inositol-3-phosphate synthase	LOC10343697	62	3.76	-1.87	10	132	P:inositol biosynthetic process; P:phospholipid
MD14G109270	calmodulin-7-like isoform X2	LOC10341012	75	4.12	-1.87	13	155	P:calcium-mediated signaling
MD12G118710	lipid transfer protein precursor	LOC10341391	75	2.47	-1.88	10	155	P:lipid transport
MD16G116000	major allergen Mal d 1-like	LOC10342396	75	3.38	-1.90	3	132	P:defense response; P:abscisic acid-activated
MD07G117480	caffeoylshikimate esterase-like	LOC10342100	68	3.36	-2.10	5	132	F:lipase activity
MD16G112010	non-specific lipid transfer protein GPI-	LOC10340317	75	2.23	-2.20	8	132	P:lipid transport; P:cuticle development;
MD08G109730	ribosome-inactivating protein	LOC10342112	62	3.50	-2.28	26	132	P:negative regulation of translation
MD16G120860	40S ribosomal protein S15-4	LOC10340866	75	2.59	-2.40	2	155	P:translation
MD03G129070	bifunctional epoxide hydrolase 2	LOC10341914	62	3.26	-2.43	3	132	
MD13G116070	major allergen Pru av 1-like	LOC10342396	62, 75	2.93	-2.45	5	132	P:defense response; P:abscisic acid-activated
MD13G111790	flavonoid 3',5'-methyltransferase-like isoform	LOC10340315	68, 75	2.90	-2.47	12	132	
MD14G124100	Calmodulin	LOC10340396	75	2.95	-3.17	5	155	P:calcium-mediated signaling

3.3.2 Cultivar 'Gala'

The Student`s t-Tests performed at each sampling date resulted in 53 proteins that were significantly different in abundance between bud samples from 'Gala' 'On' and 'Off' trees during at least one sampling date, whereas this was the case for one protein at two sampling dates (Table 2 and Annex 1). Fourteen were higher abundant in buds from 'On' trees and 39 were higher abundant in buds from 'Off' trees. Overall, significant treatment differences in abundance of proteins existed with 27 proteins at 83 dafb, one protein at 89 dafb and at 97 dafb, respectively, 21 proteins at 104 dafb and 4 proteins at 118 dafb. Cluster analysis presents four clusters of protein species, showing distinct time-dependent abundance profiles (Figure 6). Cluster 22 and 37 consisted of proteins that were more abundant in buds from 'Off' trees, meaning that those proteins could possibly be involved in floral bud development, whereas cluster 45 and cluster 49 consisted of proteins that were more abundant in buds from 'On' trees and possibly be involved in promoting vegetative growth.

Cluster 22 contains 23 proteins that were more abundant at all sampling points in buds from 'Gala' 'Off' trees than those from 'On' trees with an average log2FC of -1.66 (Figure 6). The abundance stayed relatively constant in buds from 'Off' trees but was more inconsistent with a noticeable decrease at 83 dafb and 104 dafb in buds from 'On' trees. The protein with the highest log2FC in cluster 22 was the caffeoyl-CoA 3-O-methyltransferase (MD13G1117900, EC:2.1.1.104) with log2FC of -3.13 at 118 dafb. This protein is part of the phenylpropanoid and flavonoid biosynthesis pathways (Ogata et al. 1999) and reported to be involved in the biosynthesis of lignin (Guo et al. 2001). STRING analysis of cluster 22 shows five functional clusters. The largest, with five proteins, is similar to cluster 132 in 'Fuji' and includes key enzymes of the phenylpropanoid and flavonoid biosynthetic process: PAL1, F3H, LDOX, CCOAMT and additionally trans-cinnamate 4-monooxygenase (C4H) that synthesizes p-coumaric acid in the phenylpropanoid pathway (Winkel-Shirley 1999). Furthermore, the FDM2/IDN2 complex found in cluster 22 is required for gene silencing by RNA (Böhmdorfer et al. 2014).

Cluster 37 contains 16 proteins with an average log2FC of -1.7 and was always more abundant in buds from ‘Gala’ ‘Off’ than those from ‘On’ trees (Figure 6). The abundance decreased distinctly in ‘On’ trees at 83 dafb, recovered until 97 dafb when it continuously declined toward the last sampling date. The protein with the highest log2FC in cluster 37 is the eukaryotic translation initiation factor 3 subunit F-like (MD01G1148100, P:translational initiation; F:translation initiation factor activity) with a log2FC of -2.7 at 83 dafb, which was shown to be required for embryogenesis and cell differentiation in *Arabidopsis thaliana* (Xia et al. 2010). STRING analysis of cluster 37 shows three functional clusters. The largest cluster with five proteins is a photosynthesis cluster with no relation to flower bud development. One cluster has the translation initiation factors EIF2 and EIF4A-2, which are involved in cell growth and differentiation during embryogenesis (Xia et al. 2010). There is also a cluster of two proteins involved in the methionine biosynthetic process (ATMS1 and AKHSDH2) that could be involved in the same process as the two SAMS from the ‘Fuji’ cluster 132, which has a similar abundance in ‘On’ and ‘Off’ buds.

Cluster 45 contains 9 proteins with an average log2FC of 1.9 and had a steadily increasing abundance in ‘Gala’ ‘On’ buds during the eight sampling dates (Figure 6), indicating that those proteins are possibly involved in vegetative bud growth. The separation between ‘On’ and ‘Off’ buds was occurring from the first sampling date, and prior to the calculated onset of bud initiation (Kofler et al. 2019) at 76 dafb. The protein with the highest log2FC is ferritin-4 (MD12G1178500, P:iron ion transport; P:cellular iron ion homeostasis; F:ferric iron binding) with a log2FC of 4.09 at 118 dafb. Similar to FER1 in cluster 90 in ‘Fuji’, the increase of ferritin-4 in ‘Gala’ ‘On’ buds possibly promotes growth of vegetative buds (Ravet et al. 2009). No functional clusters could be identified using STRING analysis.

Cluster 49 contains 5 proteins with an average log2FC of 1.52 and showed minor treatment differences throughout the observation period, except for a pronounced increase in ‘On’ buds at 83 dafb (Figure 6) indicating a possible involvement in vegetative bud growth. The highest log2FC (2.13) contained in cluster 49 was found at 104 dafb and is related to a class 1 heat shock protein (MD17G1020300). However, the protein with the highest log2FC (1.9) at 83 dafb

was a histone deacetylase (MD11G1156500). Histone deacetylases are reported to regulate flowering time in *Arabidopsis thaliana*, where the lack of histone deacetylases leads to a hyperacetylation of histones in the chromatin of the floral repressor FLC and thus up-regulation of FLC and delayed flowering (He et al. 2003). RNA of this protein was also reported to be upregulated in terminal buds from 'On' trees as early as 30 dafb in another study (Fan et al. 2018). Histone deacetylases are also reported to be associated with breaking seed dormancy in *Arabidopsis thaliana* (Yano et al. 2013). It could be assumed, that histone deacetylase has a vegetative growth inducing effect and initiates the growth of vegetative buds. Furthermore, histone deacetylases are often linked with transcriptional repression (Berger 2007), which may result in an inhibiting effect on floral initiation. The time point also coincides with the marked decrease of cluster 37 in 'On' buds, containing the eukaryotic translation initiation factor 3 (MD01G1148100), which was shown to be required for cell differentiation in *Arabidopsis thaliana* (Xia et al. 2010). No functional clusters could be identified using STRING analysis.

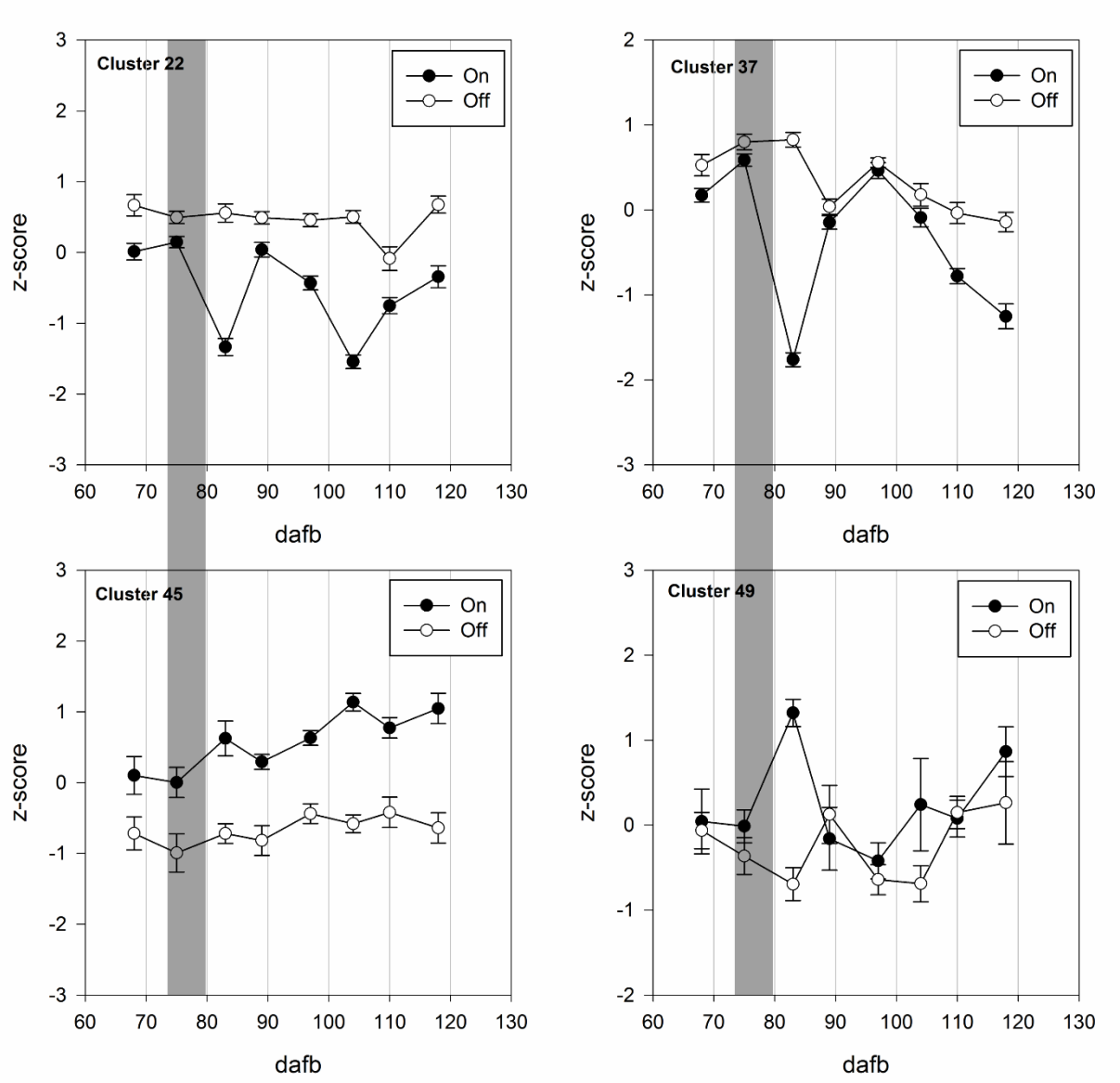

Figure 6 Normalized means of relative protein abundances from each cluster in buds from 'Gala' 'On' and 'Off' trees. All proteins that were used for hierarchical clustering showed a significant difference between 'On' and 'Off' during at least one time point at FDR=0.01. Error bars indicate standard error of z-scores. Grey bar indicates onset of bud initiation for 'Gala' 'Off' at 76 dafb as identified by histological analysis (Kofler et al. 2019). Cluster 22: n=23; cluster 37: n=16; cluster 45: n=9; cluster 49: n=5.

Table 2 shows the top 20 proteins that have the highest (more abundant in buds from 'On' trees) and lowest (more abundant in buds from 'Off' trees) log2FC for the cultivar 'Gala', respectively.

Table 2 The 40 key proteins with the highest log2FC between bud samples from 'Gala' 'On' and 'Off' trees with corresponding GDDH13 ID, description, gene symbol, indication at which sampling date the difference was significant at FDR=0.01, p-value, log2FC 'On' over 'Off', count of unique peptides per protein, cluster affiliation and biological process. 40 shown out of 53 (Annex 1).

ID	Description	Gene symbol	dafb*	-log(p)	log2FC on-off	Unique peptides	Cluster	GO Biological process
MD12G117850	ferritin-4, chloroplastic-like	LOC1034304	118	3.25	4.10	9	45	P:iron ion transport; P:cellular iron ion
MD07G126880	low-temperature-induced 65 kDa protein-like	LOC1034397	104	3.46	2.62	16	45	P:response to abscisic acid
MD02G114000	cold-shock protein CS120-like	LOC1034011	104	2.85	2.49	21	45	P:response to water
MD17G102030	class I heat shock protein-like	LOC1034421	104	3.97	2.13	6	49	
MD11G115650	histone deacetylase HDT1-like	LOC1034480	83	4.60	1.96	5	49	
MD03G116480	dihydropyrimidinase	---NA---	83	3.08	1.94	18	45	
MD02G114010	cold-shock protein CS120-like	LOC1034070	104	3.53	1.68	21	45	P:response to water
MD11G107080	asparagine synthetase [glutamine-hydrolyzing]	LOC1034066	89	3.58	1.55	26	45	P:asparagine biosynthetic process
MD14G115240	protein DEK-like isoform X2	LOC1034375	83	3.66	1.55	3	49	P:regulation of double-strand break
MD13G116140	major allergen mal d 1	LOC1034037	104	2.93	1.53	21	45	P:defense response; P:abscisic acid-
MD15G129480	formamidase-like isoform X1	LOC1034014	104	3.54	1.47	25	45	
MD13G107880	hypersensitive-induced response protein 2	LOC1034518	83	3.57	1.04	5	49	P:protein phosphorylation
MD10G112370	protein EARLY-RESPONSIVE TO DEHYDRATION	LOC1034269	104	4.35	1.02	23	45	
MD10G107630	NADH dehydrogenase [ubiquinone] iron-sulfur	LOC1034404	83	4.25	0.99	3	49	P:oxidation-reduction process
MD02G120730	3-phosphoshikimate 1-carboxyvinyltransferase 2	LOC1034280	104	4.32	-0.75	8	22	P:aromatic amino acid family
MD12G103300	3-oxoacyl-[acyl-carrier-protein] synthase III,	LOC1034108	104	4.40	-0.77	11	22	P:fatty acid biosynthetic process
MD06G113690	glutamate-1-semialdehyde 2,1-aminomutase 2,	LOC1034375	83	4.37	-1.04	10	22	P:tetrapyrrole biosynthetic process
MD17G101470	protoporphyrinogen oxidase, mitochondrial-like	LOC1034169	104	4.16	-1.12	7	22	P:porphyrin-containing compound
MD14G102270	cytochrome b-c1 complex subunit 9-like	LOC1034018	83	3.68	-1.14	7	37	P:mitochondrial electron transport,
MD06G110610	long chain acyl-CoA synthetase 4-like	LOC1034282	83	3.46	-1.17	13	37	
MD04G120400	probable linoleate 9S-lipoxygenase 5	LOC1034339	104	2.89	-1.62	27	37	P:oxidation-reduction process
MD11G131070	serine/arginine-rich-splicing factor SR34-like	LOC1034326	83	3.17	-1.63	11	37	
MD06G122610	uncharacterized protein LOC114825419	LOC1034245	83	3.20	-1.64	5	22	
MD11G105290	trans-cinnamate 4-monooxygenase	LOC1034187	83, 104	3.49	-1.69	15	22	P:oxidation-reduction process
MD15G124620	protein DMR6-LIKE OXYGENASE 2-like	LOC1034361	104	4.04	-1.72	7	22	P:oxidation-reduction process
MD08G116260	photosystem II 22 kDa protein, chloroplastic	LOC1034415	83	2.98	-1.73	4	37	
MD05G100380	bifunctional aspartokinase/homoserine	LOC1034252	83	2.97	-1.75	16	37	P:cellular amino acid biosynthetic
MD06G107160	anthocyanidin synthase	LOC1034202	104	3.62	-1.75	2	22	P:oxidation-reduction process
MD17G100350	glyceraldehyde-3-phosphate dehydrogenase A,	LOC1034257	83	2.92	-1.75	2	37	P:glucose metabolic process;
MD10G100980	nuclear pore complex protein NUP160	LOC1034034	83	3.09	-1.79	10	37	P:nucleocytoplasmic transport
MD02G128290	dynamin-related protein 4C-like	LOC1034074	104	3.30	-1.82	25	22	
MD04G117360	lipid transfer protein precursor	LOC1034535	97	3.85	-1.89	6	22	P:lipid transport
MD03G106710	internal alternative NAD(P)H-ubiquinone	LOC1034066	83	3.13	-2.02	7	37	P:oxidation-reduction process
MD07G106870	cytochrome P450 89A2-like	LOC1034388	118	3.12	-2.07	14	37	P:oxidation-reduction process
MD05G116820	chloroplast envelope quinone oxidoreductase	LOC1034358	83	3.24	-2.31	3	22	P:oxidation-reduction process
MD11G129510	ATP synthase CF1 beta subunit	LOC1034502	83	2.73	-2.41	8	37	P:ATP metabolic process; P:proton
MD05G125920	photosystem I reaction center subunit III,	LOC1034234	83	2.68	-2.46	2	37	P:photosynthesis
MD16G120140	ATP sulfurylase 1, chloroplastic	LOC1034039	118	3.35	-2.63	13	22	P:sulfate assimilation
MD01G114810	eukaryotic translation initiation factor 3 subunit F-like	LOC1034551	83	3.75	-2.70	3	37	P:translational initiation
MD13G111790	probable caffeoyl-CoA O-methyltransferase	LOC1034031	118	2.89	-3.13	12	22	

3.3.3 Comparison between cultivars

As shown in Figure 7, the correlation coefficients between the proteomic profiles of buds from ‘On’ and ‘Off’ trees decrease markedly after the calculated onset of bud initiation in ‘Off’ trees. The decrease in ‘Gala’ coincides with the postponed calculated onset, confirming the later onset on a proteomic level.

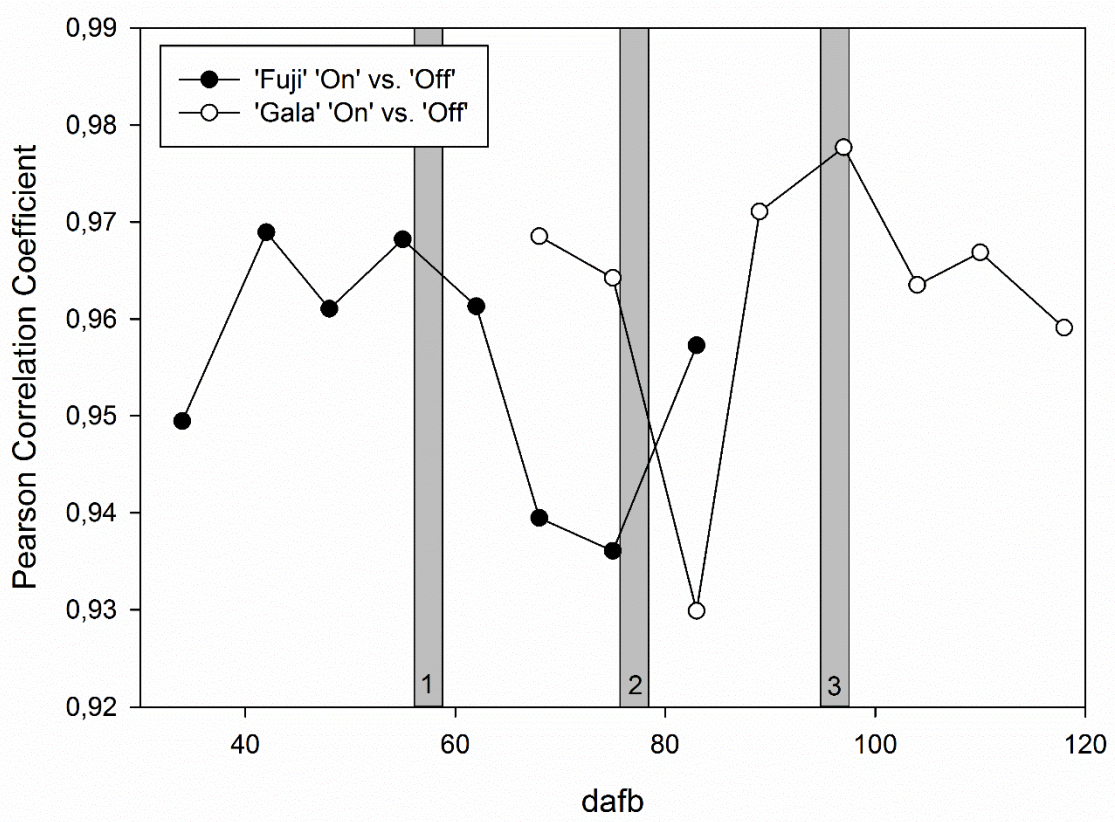

Figure 7 Pearson correlation coefficients between proteomic profiles of buds from ‘On’ and ‘Off’ trees. Grey bars indicate the calculated onset of bud initiation in ‘Off’ trees based on histological data (Kofler et al. 2019) for ‘Fuji’ (1) and ‘Gala’ (2) at 57 and 76 dafb, respectively, and for ‘Gala’ ‘On’ trees (3) at 96 dafb. No starting point of bud initiation could be calculated for ‘Fuji’ ‘On’ due to a lack of initiated buds.

As the floral bud development in ‘Gala’ trees begins later than in ‘Fuji’ trees, samples from different sampling points were compared. According to histological analysis (Kofler et al. 2019), onset of bud initiation in ‘Fuji’ ‘Off’ was at 57 dafb and in ‘Gala’ ‘Off’ at 76 dafb. In order to compare cultivar differences, it was decided to compare samples from the sampling dates prior to the calculated onset of bud initiation. Therefore, the ‘Off’ samples from 55 dafb were selected from ‘Fuji’ and from 75 dafb from ‘Gala’, respectively. The Venn diagram in figure 8 shows the occurrence of similar and exclusive proteins in each cultivar.

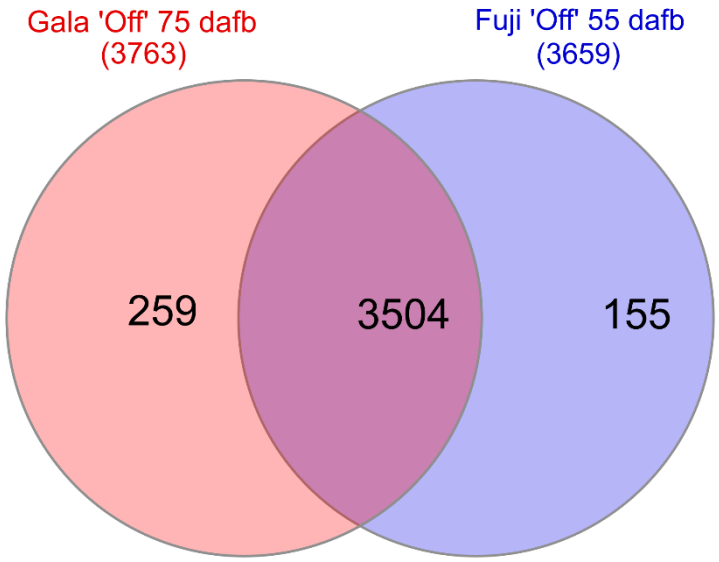

Figure 8 Venn diagram of identified proteins in buds from 'Gala' 'Off' at 75 dafb and 'Fuji' 'Off' at 55 dafb that were used for the cultivar comparison.

A Student`s t-Test, identical to the tests in chapter 3.1 and 3.2, between the two groups showed eighty-nine proteins that were significantly different abundant (Annex 2). 27 proteins were higher abundant in buds from 'Fuji' 'Off' trees, whereas 62 proteins were higher abundant in buds from 'Gala' 'Off' trees.

STRING analysis of the 27 proteins that were higher abundant in 'Fuji' than in 'Gala', identified a functional cluster consisting of flavone 3'-O-methyltransferase 1 (AT5G54160.1, OMT1), dihydroflavonol reductase (AT5G42800.1, DFR) and a putative caffeoyl-CoA O-methyltransferase (AT1G67980.1, CCOAMT). Another functional cluster contained the 60S ribosomal protein L13 (AT3G49010.3, BBC1), ribosomal protein L4/L1 (AT3G09630.1) and the 60S ribosomal protein L3-2 (AT1G61580.1, RPL3B) indicating higher translational activity in the bud meristems from 'Fuji' 'Off' trees. Furthermore, an uncharacterized protein was found to be higher abundant in buds from 'Fuji' trees: MD09G1079600, which has an 83% sequence similarity to a late embryogenesis abundant (LEA) protein from *Pyrus ussuriensis x Pyrus communis* (KAB2634038.1). LEA proteins are involved in seed maturation (Delseny et al. 2001), which could indicate an early beginning of the floral bud development in 'Fuji' 'Off'. STRING analysis of the 62 proteins that were higher abundant in 'Gala' 'On' trees showed primarily a cluster of seven heat shock proteins and a cluster of three glutathione s-transferases that could not be attributed to any floral bud development mechanisms.

3.3.4 The phenylpropanoid and flavonoid pathway

Key proteins of the phenylpropanoid and flavonoid pathways such as PAL1, C4H, F3H, LDOX showed lower abundances in buds from ‘On’ trees in both cultivars (except C4H in ‘Fuji’). The identified proteins are illustrated in Figure 9.

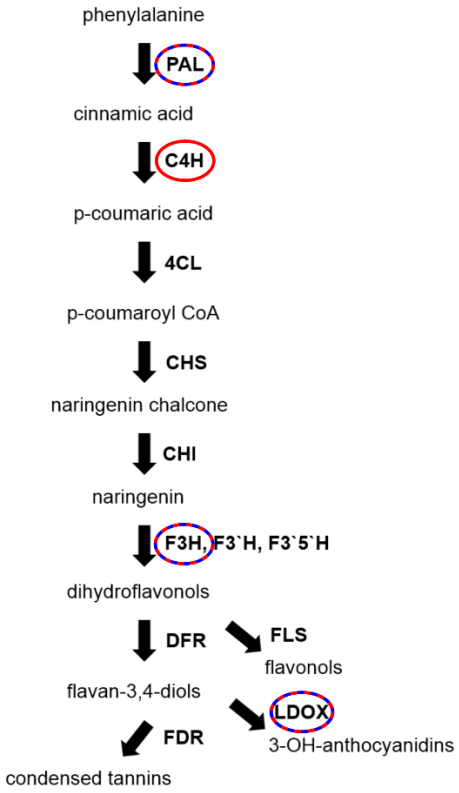

Figure 9 Schematic phenylpropanoid and flavonoid pathways, adapted from Winkel-Shirley (Winkel-Shirley 1999). Enzymes found in ‘Gala’ are marked in red, enzymes found in ‘Fuji’ are marked in blue. PAL, phenylalanine ammonia-lyase; C4H, trans-cinnamate 4-monooxygenase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, Naringenin,2-oxoglutarate 3-dioxygenase; F3`H and F3`5`H, flavonoid 3` and 3`5` hydroxylase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; FDR, flavan-3,4-diol reductase; LDOX, leucoanthocyanidin dioxygenase. Not shown is CCOAMT, caffeoyl-CoA O-methyltransferase.

One of the products of the phenylpropanoid pathway, chlorogenic acid, is correlated with the expression of PAL (Payyavula et al. 2015). It is reported, that chlorogenic acid inhibits the activity of indole-3-acetic acid-oxidase and thereby maintains the active form of auxin (Pilet 1964), a plant hormone, that, at low concentrations, encouraged bud formation in *Plumbago indica* (Nitsch and Nitsch 1967).

3.4. Conclusion

The results of this study highlight several mechanisms possibly involved in early floral bud development, during bud initiation, in apple on a proteomic level. We have identified several proteins, that show different abundances in apple buds over the period of flower bud initiation. Furthermore, proteins were identified that possibly contribute to the differences in floral bud development of apple cultivars with regular and alternate bearing behavior. These results may lay the foundation for future development of biomarkers, that help determine the degree of alternate bearing behavior early in breeding programs to select for more regular bearing cultivars.

Firstly, the lower abundance of key proteins of the phenylpropanoid and flavonoid pathway, specifically the reduced abundance of PAL, could lead to less active auxin because of the reduced production of chlorogenic acid and thereby inhibiting flower bud formation as explained in chapter 3.4. Future metabolic studies on buds from ‘On’ and ‘Off’ trees could further evaluate this hypothesis by determining the content of metabolites such as chlorogenic acid in the buds. Secondly, ferritins increased in ‘On’ buds from ‘Gala’ and ‘Fuji’ trees. The iron-storage proteins (Briat 1996) apparently play an essential role in building up enough iron storage capacities to enable vigorous vegetative growth (Ravet et al. 2009). Thirdly, the higher abundance of the histone deacetylase MD11G1156500 in buds from ‘Gala’ ‘On’ trees compared to ‘Off’ trees supports the hypothesis, that histone modification plays an essential role during flower initiation also in apple, a result that so far was described for *Arabidopsis thaliana* (He et al. 2003; Kim et al. 2013), apple (Fan et al. 2018) and rice (Shi et al. 2015). The presence of S-adenosylmethionine synthetases in ‘Fuji’ ‘Off’ buds indicate that histone and DNA methylation could possibly be involved in the flower bud induction process (Li et al. 2011). However, further epigenomic studies are necessary to confirm a causal relationship.

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Author contribution statement

JK, AM, HF and JW conceptualized and conducted the experiment; BW carried out the MS analysis; JP assisted in the statistical evaluation of the dataset; JK wrote the first draft of the manuscript; AM wrote sections of the manuscript; JW, HF and JP critically revised the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

4. Towards Systematic Understanding of Flower Bud Induction in Apple: a Multi-Omics Approach

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Anton Milyaev[1], Julian Kofler[1], Iris Klaiber[2], Stefan Czemmel[3], Jens Pfannstiel[2], Henryk Flachowsky[4], Dario Stefanelli[5], Magda-Viola Hanke[4], Jens-Norbert Wünsche[1]

[1]Institute of Crop Science, Section of Crop Physiology of Specialty Crops (340f), University of Hohenheim, Stuttgart, Germany
[2]Mass Spectrometry Unit, Core Facility Hohenheim (640), University of Hohenheim, Stuttgart, Germany
[3]Quantitative Biology Center (QBiC) Tübingen, University of Tübingen, Tübingen, Germany
[4]Institute for Breeding Research on Fruit Crops, Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Dresden, Germany
[5]Agriculture Victoria, Department of Jobs, Precincts and Regions, Bundoora, Victoria, Australia

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Abstract
Flower bud induction in apple (*Malus ×domestica* Borkh.) is tightly connected to biennial bearing, which is characterized by alternating years with high (ON) and low (OFF) crop loads. In order to study this irregular cropping behavior, spur buds from ON- and OFF-trees of the biennial bearing cultivar 'Fuji' and the regular bearing cultivar 'Gala' we collected to precisely determine the time of flower initiation. Moreover, the physiological and molecular mechanisms leading to this event were evaluated over four weeks by a multi-omics approach, including RNA sequencing, proteomic and metabolic profiling. Gene and protein enrichment analysis detected physiological pathways promoting and inhibiting flower bud initiation. The results suggest that thiamine, chlorogenic acid and an adenine derivative are involved in early flower bud development. Tryptophan showed higher abundance in spur buds collected from ON-trees than those from OFF-trees. Cultivar comparison indicated that chlorogenic acid was more abundant in 'Gala' than in 'Fuji' spur buds, whereas the opposite was found for tryptophan. Genes controlling tryptophan biosynthesis were not affected by ON- and OFF-treatments but genes assigned to the metabolism of tryptophan into idoleacetate were differentially expressed between cultivars and treatments. The multi-omics approach permitted analyzing complex plant metabolic processes involved in flower bud induction and tracing some pathways from gene-to-product level.

Citation:
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4.1 Introduction

Flower induction, initiation and differentiation are developmental stages that vegetative buds need to undergo on their way to become floral. Flower induction is commonly defined as a moment when a vegetative bud meristem perceives a signal to develop new tissue structures - flower meristems. In contrast, flower initiation is characterized by distinct morphological, microscopically visible, meristematic changes in the bud (Foster et al. 2003; Hanke et al. 2007). While flower bud initiation can be determined by histological sectioning, the exact time of flower induction still remains obscure in many plant species such as apple (*Malus* ×*domestica* Borkh.). Based on mRNA expression data of flowering genes in apple shoot apices it was assumed that the bud transition from induction to initiation could take around two weeks (Hanke et al. 2007). Early attempts to study flower initiation in apple were broadly reviewed by Jonkers in 1979 and Monselise and Goldschmidt in 1982. Merging the accumulated knowledge, some authors proposed hypothetical models of flower induction (Xing et al. 2015b; Zuo et al. 2018); however, none of them could fully explain the genetic and physiological basis of this plant developmental stage. Understanding flower induction is of great importance for controlling crop load in important perennial crops in order to establish regular annual cropping levels and to alleviate the production constraints associated with biennial bearing. The term biennial (or alternate) bearing in horticultural crops describes erratic yields when flowering density and in turn crop load in a given year strongly depends on the crop load of the previous year. It is frequently triggered by adverse environmental conditions, such as spring frost accompanied with flower damage, resulting in an OFF-year with low yield, followed by an ON-year with high yield of small-sized fruit before the repetitive cycle commences again with an OFF cropping status (Wünsche and Ferguson 2005b).

Initially, the reduced flower induction rate on high yielding apple trees was explained by sink-source interactions between fruit and buds concurrently developing within the growing season, with fruit being a stronger sink, thereby attracting more carbohydrate and in turn depriving the buds of the phloem-derived nutrients (Lenz 1979). Numerous experiments confirmed the importance of carbohydrate supply for flower bud induction *in vitro*. Among the different sugars

that have been tested in culture media, sucrose appeared to be the most effective to induce growth and development of buds (Nitsch and Nitsch 1967; Jana and Singh 2011). Moreover, it was found that plant hormones and hormone-like acting compounds are strongly involved in the flower bud induction processes. In *Plumbago indica*, *in vitro* bud formation from callus was achieved in the presence of cytokinins and adenine and further promoted by adding indole-3-acetic acid (IAA). In the same study, flower bud formation was inhibited by application of three different gibberellins (Nitsch and Nitsch 1967).

Recently developed omics analytical strategies have been driven largely by technological advances in mass-spectrometry for proteomics and metabolomics and next generation sequencing (NGS) assays, including RNA-Seq for transcriptomics. These high-throughput methods are cost-effective and target specific classes of biomolecules such as RNA transcripts, proteins and primary or secondary metabolites. Despite the immense amount of data that can be obtained using each of those approaches, implementing only one of them, as for example done by Li *et al.* (2016) and Li *et al.* (2019) in apple and by Muñoz-Fambuena *et al.* (2013) in citrus, might not be sufficient to understand complex biological mechanisms such as flower induction in plants. With the appearance of gene detection techniques, considerable research was devoted to the discovery of genes and transcription factors, which may promote or suppress flowering. Indeed, dozens of sequences were initially described as flowering regulators in Arabidopsis and later confirmed to be present as homologs in apple (Flachowsky et al. 2010, 2012; Haberman et al. 2017).

Despite these achievements in genomics, the current knowledge about the proteome and metabolome of apple is very limited and described so far only in the context of fruit development and maturation by Lin and Harnly (2013), Buts *et al.* (2016) and Li *et al.* (2016). Specifically, proteomic and metabolic data sets of apple buds in relation to biennial bearing are still missing. Many authors studied flower bud development by looking at the activity of particular genes (Zuo et al. 2018), transcription factors (Vimolmangkang et al. 2013) and proteins (Foster et al. 2007), which had already been discovered in other plants. To study the underlying biological processes involved in flower induction and to trace them from gene to

product, it is necessary to combine several omics approaches in an attempt to better understand the interplay between genes, proteins and metabolites determining the reproductive development of plants. The target and the novelty of the current work is the application of NGS in combination with two non-targeted omics approaches in order to study metabolic pathways promoting and inhibiting flower induction, to detect any possible molecular signals involved in flower bud development in apple and to link all the compounds of interest to genes and proteins involved in their biosynthesis and regulation.

In the same experimental setup, we sampled apple buds for histological sectioning and revealed flower initiation time points for ‘Fuji’ and ‘Gala’ under field conditions in southwest Germany (Kofler et al. 2019). Considering these time points, we aimed to detect mobile signals promoting or inhibiting flower bud induction such as peptides, phytohormones, phytohormone-like acting compounds, sugars and secondary metabolites, which could be detectable 1-4 weeks prior to flower initiation. The unknown mobile signals could influence or could be influenced by expression patterns of genes, determining the fate of the bud meristem. In order to test this hypothesis comprehensively on the transcript, protein and metabolite level, we used a holistic multi-omics approach, targeting the flower induction mechanisms of the biennial bearing apple cultivar ‘Fuji’ and of the regular bearing apple cultivar ‘Gala’.

Apple spur buds, which were used for multi-omics analyses, were collected from ON- and OFF-trees over four weeks leading up to flower initiation, covering the assumed period of flower induction. RNA extracted from the buds was analyzed using Next Generation RNA sequencing, proteins and metabolites were detected using electrospray ionization (ESI) mass-spectrometry in order to create multi-omics profiles of apple spur buds and to reveal the differences between ON- and OFF-trees. Here we summarize the results of RNA sequencing and non-targeted proteomic and metabolic profiling.

4.2 Materials and methods

4.2.1 Plant material and experimental design

The experimental apple orchard was located at the Centre of Competence for Fruit Cultivation near Ravensburg, Germany (47°46'2.89"N 9°33'21.21"E, altitude 490m). The study was performed using 7-year-old 'Fuji' (clone 'Raku-Raku') and 'Gala' (clone 'Galaxy') apple trees (130 trees of each cultivar) grafted on M.9 rootstock. Trees of each cultivar were planted in two rows at 3 x 1 m spacing, respectively, trained as tall spindles of 3.5 m height and managed with standard irrigation and plant protection programs for the area. At the time of full bloom (30 April 2015) all flowers from randomly selected 65 trees per cultivar were removed by hand (OFF-trees), while the remaining trees were not thinned and maintained their natural flower density and hence high crop load (ON-trees). Subtending apple buds on 2-year-old spurs were collected weekly starting from four weeks after full bloom for 15 weeks until 2 September 2015. At each sampling week, 55 buds were collected from each of four randomly selected treatment-trees for proteomic and metabolic profiling and for RNA sequencing. After the brown bud scales were removed, the buds were placed into safe-lock tubes and snap-frozen in liquid nitrogen. The samples were stored at -80°C until used for analysis. The workflow including the plant material, treatments, sampling scheme and analytical procedures used in this study is shown in Figure 1.

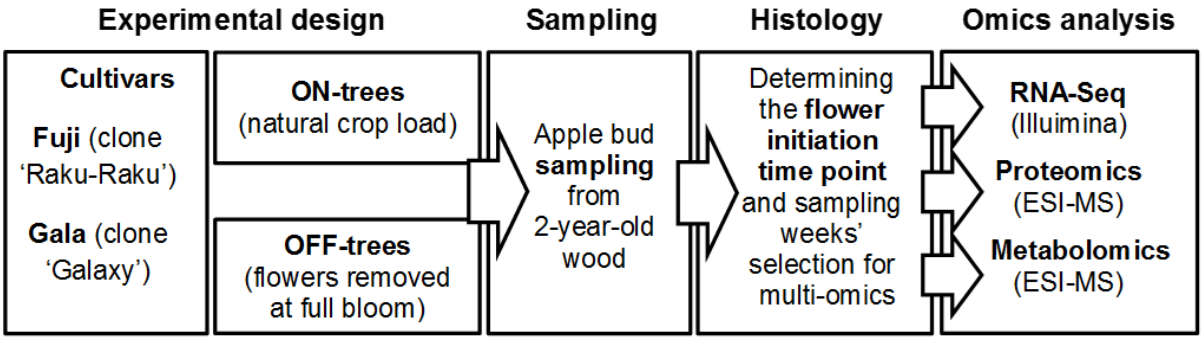


Figure 1 Schematic diagram of the plant material, treatments, sampling scheme and analytical procedures used in this study.

4.2.2 Sampling time window for the analyses

Observing flower bud meristem development by histological sectioning of apple buds sampled in the same experiment throughout the growing season of 2015, we identified flower initiation time points for ‘Fuji’ (75 days after full bloom, DAFB) and for ‘Gala’ (97 DAFB) (Kofler *et al.*, 2019). The findings presented not only microscopically observed flower initiation time points but also suggested the predicted (modelled) time of the onset of flower initiation. The sampling window selection for the current study was based on the microscopically identified flower initiation time points. Assuming that a signal for flower bud induction must be detectable at least two weeks prior to flower initiation, we selected four sampling weeks prior to this event in order to perform omics analyses and to capture triggers of flower induction. Consequently, the multi-omics analysis covers sampling dates 48, 55, 63 and 68 DAFB, corresponding to 17 June, 24 June, 01 July and 07 July for ‘Fuji’ and 68, 75, 83 and 89 DAFB, corresponding to 07, 14, 21 and 28 July for ‘Gala’.

4.2.3 RNA sequencing

Three trees, serving as replicates, were randomly selected for each of the two treatments (ON and OFF) at each of four sampling weeks chosen for the analysis. Five out of 55 collected buds per replicate were randomly taken for RNA extraction. This was performed using the InviTrap Spin Plant RNA Mini Kit (Invitek Molecular GmbH, Berlin, Germany) according to the standard protocol with the following modifications: Samples were first ground to powder using a cryogenic mixer mill (CryoMill, Retsch GmbH, Haan, Germany) cooled with liquid nitrogen. The grinding was done in safe-lock tubes with two steel balls (Ø=5 mm) in one cycle of 10 s pre-cooling and 6 min grinding at 25 Hz. A mixture of mercaptoethanol and lysis solution RP (1:100) was used for cell membrane disruption. Total RNA of each sample was eluted in 40 µl RNAse-free water. The removal of contaminating DNA from the RNA preparations was achieved with the DNA-free DNA Removal Kit (Thermo Fisher Scientific, Waltham, USA). All the RNA samples were stored at -80 °C until required.

RNA sequencing was conducted with c.ATG at the University of Tübingen, Germany. All samples were sequenced on a HiSeq2500 (Illumina machine, Illumina Inc., San Diego, USA) using paired-end (PE) mode and producing ~10 million reads per sample. The resulting fastq files were demultiplexed and transferred to QBiC and analyzed on an HPC cluster of the University of Tübingen in a fully automated way using a Nextflow-based RNA-Seq pipeline (https://github.com/nf-core/rnaseq, release 1.3). At the core of this workflow, *FASTQC* v0.11.8 (Andrews 2010) was used to determine quality of the FASTQ files. Subsequently, adapter trimming was conducted with *Trim Galore* v0.5.0 (Krueger et al. 2012). *HISAT2* (v2.1.0) aligner was used to map the reads that passed the quality control against the GDDH13 apple genome version 1.1 from The National Institute of Agricultural Research (INRA) (https://iris.angers.inra.fr/gddh13/) (Daccord et al. 2017). Annotation and fasta file were downloaded from there in January 2019. Read counting of the features (e.g. genes) was done with featureCounts v1.6.4 (Liao et al. 2014). For differential expression analysis the raw read count table resulting from featureCounts was fed into the R package *limma* (v. 3.32.10) and *edgeR* (v. 3.18.1). First, the raw read count table was filtered for genes that had no expression in any of the samples. The remaining counts were then normalized by sequencing depth and log2-transformation using the edgeR functions calcNormFactors() and cpm() in order to meet the assumptions of linear models. To identify differentially expressed genes (DEGs) at each time point between 'Fuji' ON and 'Fuji' OFF as well as between 'Gala' ON and 'Gala' OFF conditions, a simple linear model was fitted to each gene consisting of a fixed effect for a combined factor of time (levels: 48, 55, 63, 68 DAFB for 'Fuji' and 68, 75, 83, 89 DAFB for 'Gala') , genotype (levels: 'Fuji' and 'Gala') and treatment (levels: ON and OFF). *Limma* was then also used to extract pairwise contrasts including statistics for each gene including empirical Bayes moderated p-values which were finally adjusted for multiple testing by controlling the false discovery rate (FDR) using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). As threshold, a gene was called a differentially expressed gene (DEG) with a multiple adjusted p value ≤ 0.05%. No log fold change filter criterion was applied for statistical assessment to find DEGs. For exploratory analysis, counts were normalized using

the *DESeq2* package (v. 1.16.1) and visualized using standard packages in R (version 3.4.0). Translation of gene IDs into gene symbols was made using Blast2GO software (Conesa et al. 2005), where both apple genomes (GDDH13 apple genome version 1.1 published by INRA and MalDomGD1.0 apple genome published by The National Center for Biotechnology Information) were aligned. Gene symbols corresponding to gene IDs were chosen from the best match column. For gene enrichment analysis and gene mapping to KEGG pathways, we used gene annotation software KOBAS 3.0 (Xie et al. 2011). All enriched pathways presented in the current work were selected from the main result output according to the p value ≤ 0.05%. Venn diagram was created using online software InteractiVenn (Heberle et al. 2015). Raw data can be accessed on ArrayExpress with the dataset identifier E-MTAB-9644 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9644/).

4.2.4 Proteomic profiling

Three out of the four trees per treatment and sampling week were randomly selected for proteomic profiling. Each replicate consisted of 16 buds, which were randomly taken from each 55-bud sample per tree. Sample preparation and analysis were performed according to (Kofler *et al.* 2020). In order to compare ON- and OFF-treatments, relative quantification of proteins was performed.

Reverse hits, proteins identified only by site and potential contaminants were removed from the dataset, LFQ intensities were log(2) transformed, rows were filtered based on a minimum of three out of six potential valid values at each sampling date and a minimum of two unique peptides per protein. In order to perform a principal component analysis (PCA), missing values were imputed by random numbers from the normal distribution and a down shift of 1.8. For other statistical tests, missing values were not imputed, however all the proteins found only in spur buds collected from ON- or only in the buds collected from OFF-trees, were taken for enrichment analysis and KEGG search. A two-sided Student T-test at each date between the treatments was calculated with S0 = 0.1 and a permutation-based false discovery rate (FDR) of 0.05. Proteins which were statistically significant according to the T-test and exhibited a log2

fold change > 0.5 were considered as differentially abundant. Differentially abundant proteins (DAPs) between treatments in at least one sampling week were considered for the subsequent KEGG pathway analysis. Raw data can be accessed on ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021716 (https://www.ebi.ac.uk/pride/).

4.2.5 Metabolic profiling

For metabolic profiling, one bud from each out of four trees per treatment at each of 4 sampling weeks was analyzed. Each bud was randomly taken from a mixture of 55 frozen buds collected from one tree representing one out of four replicates.

Metabolites from each bud were extracted in a separate safe-lock tube. Since the buds and safe-lock-tubes had a slight weight deviation, all the empty tubes (64 in total) were numbered and weighed on the analytical balance to standardize the extraction conditions by adjusting the solvent volumes according to the net sample weight. Frozen buds were then quickly placed into corresponding tubes, weighed again in order to calculate net weights of the samples, opened to put in a pre-cooled steel ball (Ø=5 mm) and immediately returned to liquid nitrogen. The samples were ground to powder using a CryoMill (Retsch GmbH, Haan, Germany) at a frequency of 20 Hz with 10 s pre-cooling and 4 min grinding. Each sample was eluted with ice-cold (-20 °C) solution of 80% methanol and 20% distilled water, vortexed for 10 s and placed on ice. The samples were kept frozen until cold 80% methanol was added. Methanol volumes were adjusted for each sample by using 120 µl methanol (80%) per 1 mg bud fresh weight. Steel balls were removed from the tubes using a magnet and extracts were left at -20 °C for 24 h for incubation. Incubated samples were centrifuged at 10,000 rcf for 4 min, the supernatant containing metabolites was transferred into new tubes, which were kept at -20 °C until required.

Non-targeted metabolic profiling was carried out at the Mass-Spectrometry Core Facility Unit at the University of Hohenheim, Stuttgart, Germany, using ultra-high-performance liquid chromatography (UHPLC) coupled with electro-spray ionization mass spectrometry (ESI-MS).

The UHPLC unit Agilent 1290 Infinity LC System (Agilent Technologies, Inc., Santa Clara, USA) was equipped with an Acquity CSH C18 1.7 µm, 2.1 x 150 mm column (Waters Corporation, Milford, USA). Sample components were separated under the column temperature of 40 oC using 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) as solvents. From each extract, we used 7 µl for UHPLC injection with flow rate of 400 µl min-1 and the following gradient: at 0 min 97% A and 3% B, from >0 to 15 min 80% A and 20% B, from >15 to 40 min 5% A and 95% B and from >40 to 44 min 97% A and 3% B. Mass-spectrometry was performed on a Thermo Scientific Q-Exactive Plus Orbitrap System (Thermo Fisher Scientific, Waltham, USA) in positive and negative ionization modes. Spray capillary voltage was set at 4.2 kV in positive mode and at 3.5 kV in negative mode, desolvation temperature was 380 oC. Mass spectra were acquired using a scan range from 140 to 1500 m z-1 at a resolution of 70000 full width half maximum (FWHM), an Automatic Gain Control (AGC) target of 1.0 × 106 of and 100 ms maximum ion injection time. Data dependent MS/MS spectra in a mass range of 200 to 2000 m/z were generated for the five most abundant precursor ions with a resolution of 17500 FWHM using an AGC target of 5.0 × 104 of and 64 ms maximum ion injection time and a stepped collision energy of 15, 30, and 55 V. The measurement was started with a blank solution, after which the extracts (samples) were injected in a randomized order. Quality control (QC) samples (a mixture of all the samples in equal proportions) were injected after every 10 measurements. Reference compounds were obtained from Merck KGaA (Darmstadt, Germany).

In order to compare ON- and OFF-treatments, relative quantification of metabolites was performed. For high-resolution accurate-mass data analysis and compound identification we used Xcalibur 4.0.27.13 and Compound Discoverer 2.1.0.398 (Thermo Fisher Scientific, Waltham, USA) software. Fragmentation spectra and molecular masses of detected compounds were aligned to the references retrieved from the databases mzCloud, ChemSpider, Plant Metabolic Network (PMN) and PubChem. Additional statistical analyses of normalized peak areas were performed with Perseus 1.6.1.3 (Tyanova et al. 2016) and online-

software MetaboAnalyst 4.0 (Chong et al. 2019). PCA including both cultivars was not possible to perform due to analysis of samples from ‘Gala’ and ‘Fuji’ as two separate batches.

4.3 Results

4.3.1 Gene expression and proteomics

Next Generation RNA sequencing of ‘Fuji’ and ‘Gala’ spur buds detected 40,916 genes (out of 46,558 total annotated genes) for which reads were successfully mapped to the double haploid ‘Golden Delicious’ GDDH13 genome version 1.1. In total, 6,967 genes in ‘Fuji’ and 3,426 genes in ‘Gala’ were differentially expressed between ON- and OFF-trees in any of the four selected sampling dates (Figure 2a, b). From those differentially expressed genes (DEGs), 1,057 were detected in both genotypes, while 5,910 DEGs were detected only in ‘Fuji’ and 2,369 DEGs only in ‘Gala’ (Figure 3a). The number of DEGs was not evenly distributed between the treatments and over the sampling dates. The majority of DEGs was observed 63 DAFB in ‘Fuji’ and 83 DAFB in ‘Gala’, corresponding to approximately 2 weeks prior to flower initiation in both cultivars (Figure 2a, b). At this developmental stage, a higher abundance of DEGs in spur buds from ‘Fuji’ OFF-trees was observed compared to ‘Fuji’ ON-trees, whereas the number of DEGs in ‘Gala’ was nearly the same in both treatments.

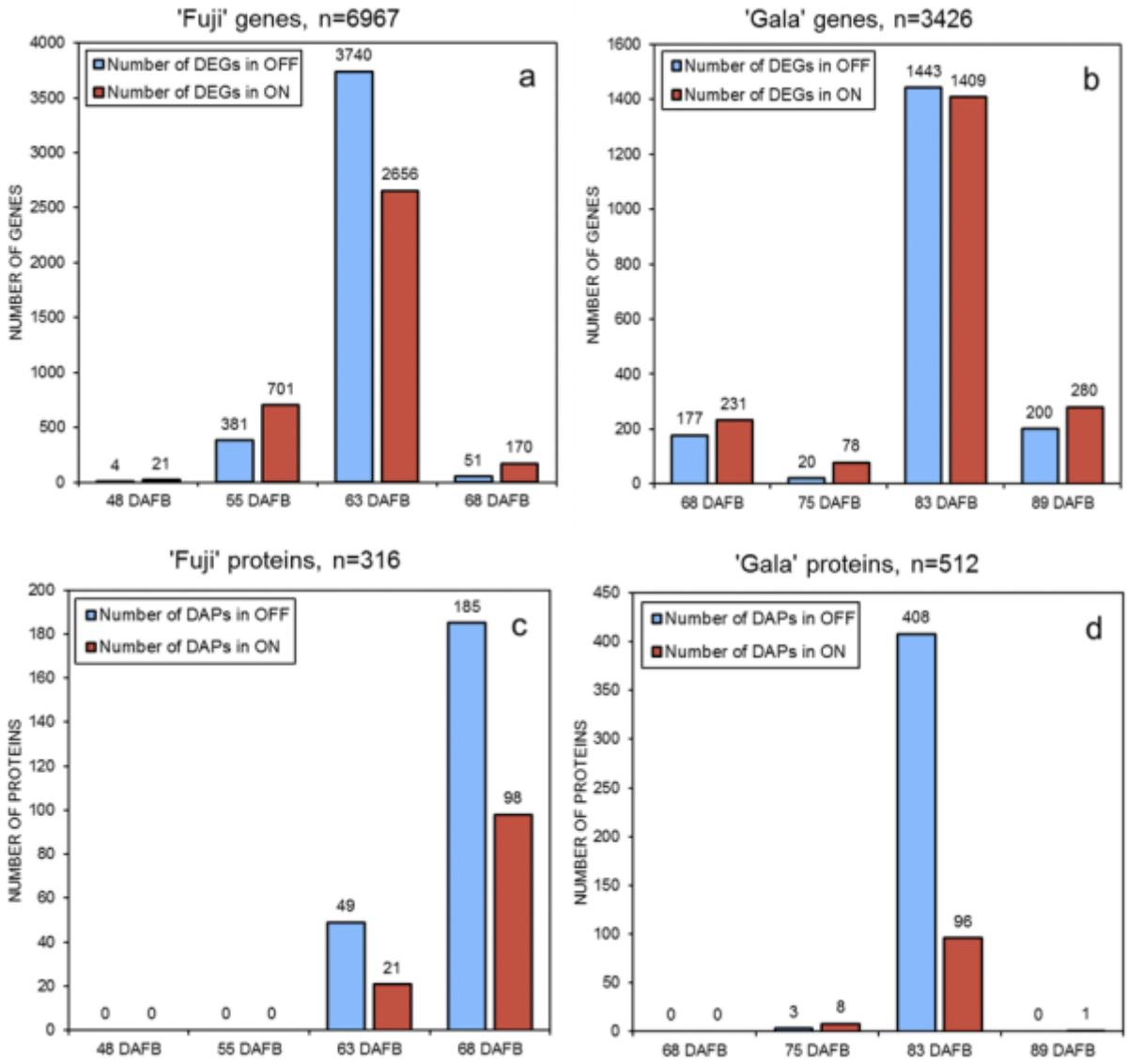


Figure 2 The number of DEGs (a, b) and proteins (c, d) in 'Fuji' (a, c) and 'Gala' (b, d) between ON- and OFF-trees over four weeks prior to flower initiation. Flower initiation in 'Fuji' occurred 75 DAFB and that in 'Gala' 97 DAFB. The sums of the bar plots are not equal to the numbers (n) shown above each plot as n indicates unique DEG IDs only.

Proteomic profiling of 'Fuji' and 'Gala' spur buds resulted in a total number of 7,121 proteins detected by mass-spectrometry. From them, 7,075 protein IDs could be linked to the corresponding genes identified in the transcriptomic analysis. Data quality filtering and statistical analyses of the proteomic profiles detected 316 differentially abundant proteins (DAPs) in 'Fuji' and 512 DAPs in 'Gala' (Figure 2c, d). These DAPs were primarily found at 63 and 68 DAFB in 'Fuji', while in 'Gala' predominantly at 83 DAFB, a period, which corresponds to 1-2 weeks prior to flower initiation in both genotypes. From the total number of DAPs, 90 were common for both cultivars (Figure 3b). Comparison of transcriptomic and proteomic

datasets showed that 141 DEGs in ‘Fuji’ and 78 DEGs in ‘Gala’ had corresponding DAPs (Figure 3c, d).

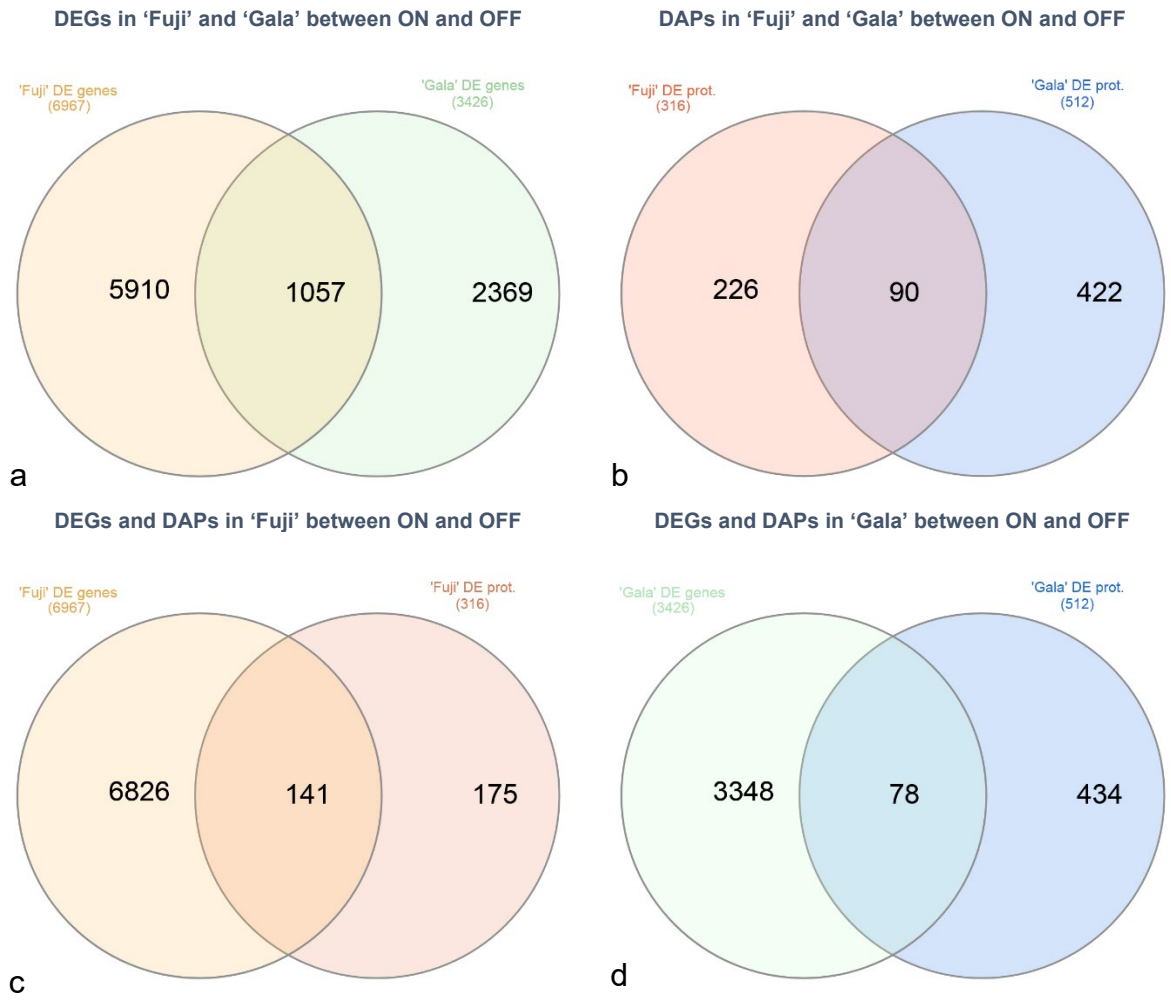


Figure 3 Number of DEGs (a) and DAPs (b) between ON- and OFF-trees overlapping in ‘Fuji’ and ‘Gala’ and comparison of DEGs and DAPs within the cultivars (c, d) 1-4 weeks prior to flower initiation.

Principal component analysis (PCA) of transcriptomic (Figure 4a) and proteomic (Figure 4b) data with variances of two main components of 31.86 and 29.03, respectively, showed clear distinctions between the studied cultivars. In the transcriptomic data we observed prominent differences between ‘Fuji’ ON- and OFF-trees 63 DAFB, whereas in the proteomic data there was a clear distinction between ‘Gala’ ON- and OFF-trees 83 DAFB. Both time points correspond to approximately two weeks prior to flower initiation in both cultivars, respectively. DEGs and DAPs were mapped to KEGG pathways using gene list enrichment analysis with Kobas 3.0 (Xie et al. 2011). Enrichment analysis showed promoting (blue) and inhibiting (red) metabolic pathways for flower bud initiation in apple (Figure 5a, b), which were overrepresented in a given gene (or protein) list compared to the genome (or proteome)

background information. Bud development mechanisms in OFF-trees (flower induction promoting pathways) included carbon fixation, fatty acid biosynthesis, purine and pyrimidine metabolism, DNA replication, biosynthesis and metabolism of amino acids, steroid biosynthesis, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, flavonoid biosynthesis and others presented in Figure 5.

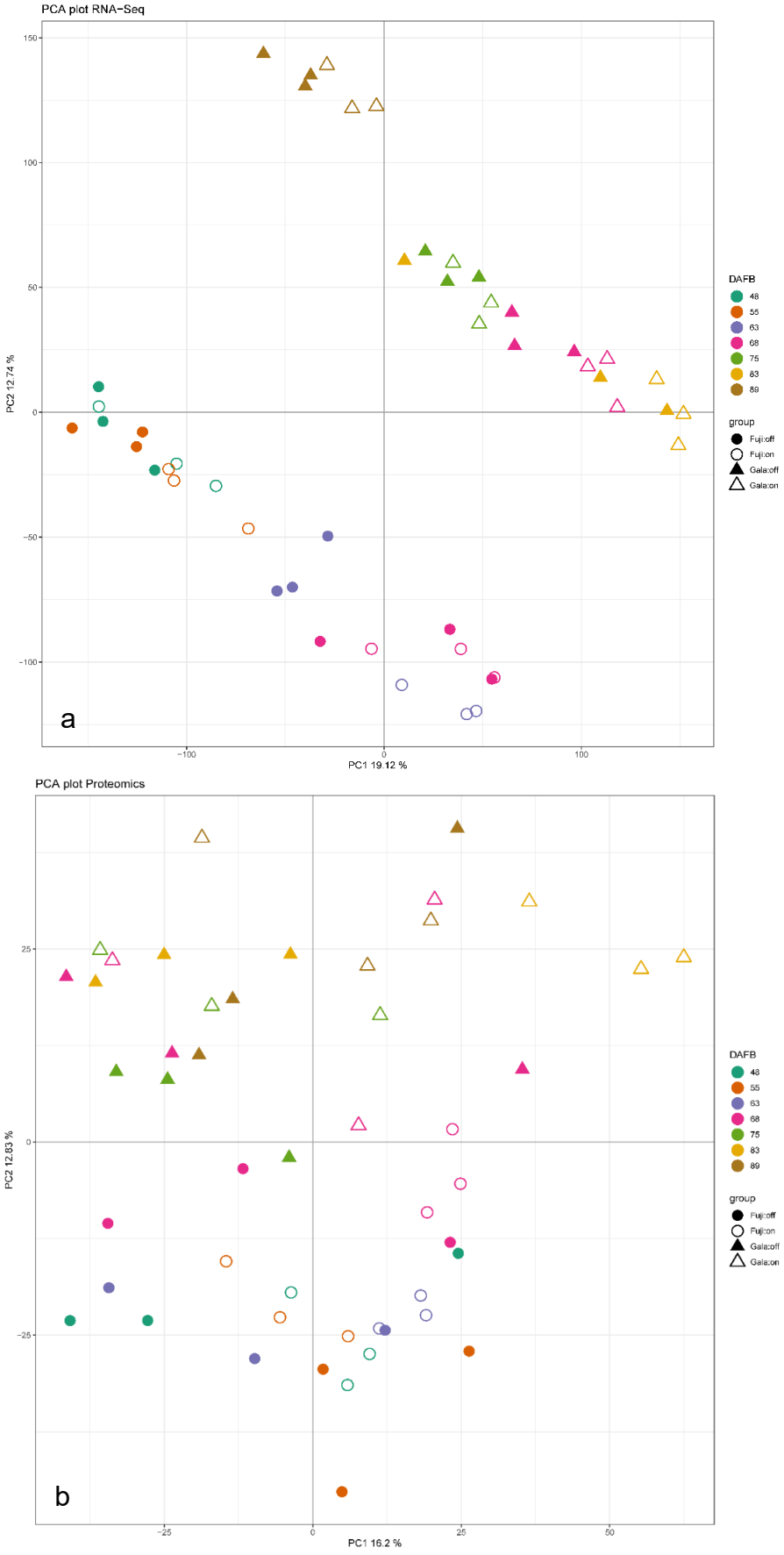


Figure 4 PCA analysis of transcriptomic (a) and proteomic (b) data.

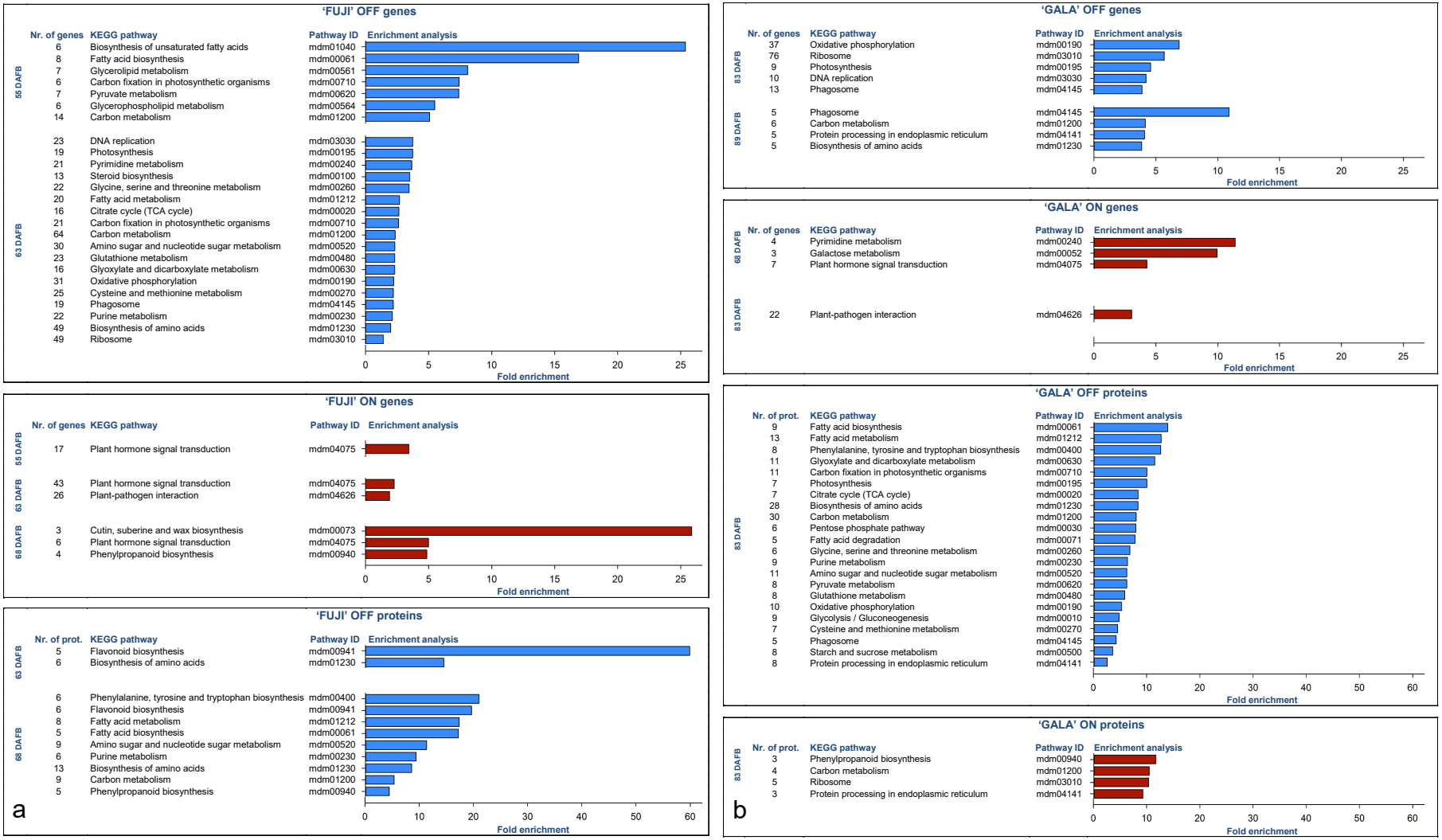


Figure 5 Enrichment analysis of KEGG pathways of DEGs and DAPs between ON- and OFF-trees of 'Fuji' (a) and 'Gala' (b). Fold enrichment was calculated by comparing the background frequency of total genes or proteins annotated to that term in Malus ×domestica to the sample frequency, representing the number of genes or proteins entered that fall under the same term (Dalmer and Clugston 2019). In 'Fuji' ON, no significantly enriched pathways of DAPs were found. Detailed information about pathway entries and statistics is provided in Supplementary Tables 1-3.

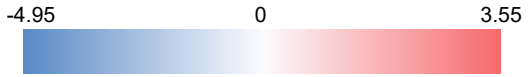
In spur buds from ON-trees of both cultivars plant hormone signal transduction pathway, plant-pathogen interaction pathway and phenylpropanoid biosynthesis were overrepresented (Figure 5a, b). Plant hormone signal transduction pathway in ‘Gala’ ON included 7 DEGs regulating signaling mechanisms of auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA) and jasmonic acid (JA), whereas in the same pathway in ‘Fuji’ ON, 46 DEGs controlling signal transduction of at least eight known phytohormone groups: auxins, CKs, JA, GAs, ABA, salicylic acid (SA), brassinosteroids (BS) and ethylene were detected (Supplementary Tables 1 and 2). Enriched pathways of DEGs and DAPs within OFF-trees had partial overlaps. More distinct were enriched pathways of DEGs and DAPs in ON-trees. Cutin, suberine and wax biosynthesis pathway was found to be enriched only in ‘Fuji’ ON and only in DEGs while there was no significantly enriched pathway based on DAPs in ‘Fuji’ ON-trees. In ‘Gala’, some metabolic pathways in ON-trees were detected, which were also found in OFF-trees of the same cultivar: Carbon metabolism, ribosome and protein processing in endoplasmic reticulum, and pyrimidine metabolism.

4.3.2 Metabolic profiling

Besides transcriptomics and proteomics analyses, we also performed metabolic profiling of apple spur buds to detect the final products of gene expression activity and analyzed the abundances of small molecules, which may serve as mobile signals to trigger or inhibit flower induction. Computation of metabolic data revealed 1,491 mass/charge-signals (features) in the positive and 796 features in the negative ionization mode. From those, 1140 unique features had MS^1 isotope patterns and fragmentation spectra (MS^2-spectra) of sufficient quality. Based on their sum formulas and tentative assignment in ChemSpider, PubChem and Plant Metabolic Network (PMN) databases we were able to obtain general information about the compound classes that could be detected in apple bud tissue. These included amino acids and dipeptides, plant hormone-like acting substances, polyphenols and their glucosides, vitamins, triterpenoids, fatty acids and unknown compounds, which are not yet included in the chemical databases. By the automated analysis of accurate m/z ratios and MS^1-isotope patterns of the

detected compounds, we obtained sum formulas for each of the metabolites. Based on the precise molecular mass search in the mentioned chemical databases and comparison of fragmentation spectra with existing reference spectra in mzCloud, 159 features could be linked to potential compounds (pre-identification step). After manual inspection of MS^1 isotope patterns and fragmentation spectra in order to avoid false positive identifications, the number of pre-identified compounds was further reduced to 111. They were characterized with robust MS^1-signals, fragmentation spectra of sufficient quality and measurable peak areas, which were used for further downstream processing.

Statistical data analysis of pre-identified 111 compounds revealed 22 compounds, which abundances differed significantly between the spur buds from ON- and OFF-trees of either one or both cultivars in at least one out of four sampling weeks (Figure 6). The metabolite identification process is considered to be completed only, if the structures of all candidate substances are confirmed by reference compounds. From those 22 compounds, 5 were available for purchasing as reference substances (prolylleucine, thiamine, chlorogenic acid, arginine, and tryptophan). These compounds were used for verification of the database search result by comparing MS^1 isotope patterns, retention times and MS^2-spectra to the corresponding features obtained by metabolite profiling. As a result, four reference compounds fully confirmed the proposed structures of thiamine, chlorogenic acid, arginine, and tryptophan, whereas prolylleucine was not confirmed.



Nr.	MS mode	Compound name (confirmed with reference compounds)	Potential candidate according to the chemical databases*	Molecular formula	Molecular weight, Da	RT, min	Compound abundance, log2FC difference between 'Fuji' ON and 'Fuji' OFF				Compound abundance, log2FC difference between 'Gala' ON and 'Gala' OFF			
							48 DAFB	55 DAFB	63 DAFB	68 DAFB	68 DAFB	75 DAFB	83 DAFB	89 DAFB
1	Neg		1-O-(4-coumaroyl)-beta-D-glucose	$C_{15}H_{18}O_8$	326.09986	8.389	2.14	2.21	3.06	3.55	1.30		2.02	1.92
2	Pos		Glucosylgalactosyl hydroxylysine	$C_{18}H_{34}N_2O_{13}$	486.20669	12.762	1.39			1.50	2.23	1.93		1.65
3	Pos	Tryptophan	Tryptophan (CONFIRMED)	$C_{11}H_9NO_2$	204.08988	3.896				1.90	1.28		1.97	
4	Pos		L-Glutamic acid	$C_5H_9NO_4$	147.05274	0.934						0.62		0.66
5	Pos		Kaempferol + 5-Sugar	$C_{15}H_{10}O_6 + C_5H_{10}O_5$	418.08923	18.304			0.70					
6	Neg		Kaempferol 3-rhamnoside	$C_{21}H_{20}O_{10}$	432.10533	18.83					-0.46	-0.46	-0.49	
7	Pos		3-trans-p-Coumaroylrotundic acid	$C_{39}H_{54}O_7$	634.38552	33.049							1.13	
8	Pos		Phloridzin + 5-Sugar	$C_{21}H_{24}O_{10} + C_5H_{10}O_5$	568.17754	16.527	-0.73	-1.07		-0.65	-0.39	-0.58		
9	Pos		Astilbin	$C_{21}H_{22}O_{11}$	450.11506	15.91	-1.39			-1.68	-0.43			
10	Pos		Phloridzin + 6-Sugar	$C_{21}H_{24}O_{10} + C_6H_{12}O_6$	598.18843	15.45	-1.13	-1.36		-0.97	-0.78	-1.18		
11	Pos	DL-Arginine	DL-Arginine (CONFIRMED)	$C_6H_{14}N_4O_2$	174.11124	0.772	-1.98	-3.34						
12	Pos	Chlorogenic acid	Chlorogenic acid (CONFIRMED)	$C_{16}H_{18}O_9$	354.09412	9.31				-2.28	-1.18	-2.05	-2.14	
13	Pos		Sieboldin	$C_{21}H_{24}O_{11}$	452.13086	15.91	-1.66	-1.31		-2.31	-0.56			
14	Pos		L-1-glycero-phosphorylcholine	C8H20NO6P	257.10192	0.973	-1.55	-1.34		-1.62				
15	Pos	Thiamine	Thiamine (CONFIRMED)	$C_{12}H_{17}N_4OS$	264.10388	0.877	-2.02		-1.39				-1.31	
16	Pos		Argininosuccinic acid	$C_{10}H_{18}N_4O_6$	290.12166	0.91	-2.40	-3.86						
17	Pos		Prolylleucine (REJECTED)	$C_{11}H_{20}N_2O_3$	228.14657	1.022	-2.20		-2.55	-2.05	-1.82	-1.65	-1.85	-1.54
18	Neg		Glycerophosphoglycerol	$C_6H_{15}O_8P$	246.05001	1.904						-1.53	-1.30	-1.17
19	Pos		Glycerophosphoinositol	$C_9H_{19}O_{11}P$	334.06573	1.749	-2.36			-2.28		-1.75	-0.99	-1.41
20	Pos		N-acetyl-L-2-aminoadipic acid	$C_8H_{13}NO_5$	203.07899	1.272	-3.42				-1.50	-2.66	-2.04	
21	Pos		Allysine	$C_6H_{11}NO_3$	145.07347	0.944	-3.75		-2.82		-1.95	-1.99	-1.72	-1.65
22	Pos		1-Methyladenine	$C_6H_7N_5$	149.06976	0.833	-4.95	-3.08	-2.83	-3.73		-3.23	-2.59	

Figure 6 Differentially abundant metabolites (DAMs) in spur buds from ON- and OFF-trees of 'Fuji' and 'Gala'. *Pre-identified metabolites according to mzCloud, ChemSpider, Plant Metabolic Network (PMN), PubChem. "CONFIRMED" indicates identified organic molecules confirmed with reference compounds, "REJECTED" indicates structure and/or retention time is not confirmed using reference compounds. Negative log2FC values (blue) indicate a higher compound abundance in OFF, positive log2FC values (red) indicate a higher compound abundance in ON. Cells of the same color on the heat map indicate the same level of log2FC metabolite abundance difference between ON- and OFF-trees and do not indicate significant differences. Numbers on the heat map specify exact log2FC differences between ON- and OFF-trees and mark significant differences at multiple adjusted p value ≤ 0.05.

4.3.3 Analysis of candidates across omics levels

For analysis downstream of the individual omics analysis, we focus on the fully confirmed compounds and also genes and proteins involved in their biosynthesis and metabolism. In the Figures 7-9, genes and proteins are shown, which are assigned to the metabolism of thiamine, chlorogenic acid and tryptophan detected in spur buds of ‘Fuji’ and ‘Gala’. Thiamine was more abundant in spur buds from OFF-trees in both apple cultivars. The compound’s abundance in ‘Fuji’ ON and ‘Gala’ ON was at similar level, whereas comparing spur buds from OFF-trees, it was slightly higher in ‘Fuji’. KEGG library indicates that thiamine originates from thiamine phosphate and can be further metabolized to thiamine diphosphate and thiamine triphosphate by thiamine diphosphokinase (EC:2.7.6.2; EC - Enzyme Commission number) and adenylate kinase (EC:2.7.4.3), respectively. The majority of DEGs and DAPs, which are assigned to those reactions, were also higher expressed in OFF-trees at 63 DAFB (‘Fuji’) 83 DAFB (‘Gala’) corresponding to 2 weeks prior to flower initiation in both cultivars (Figure 7).

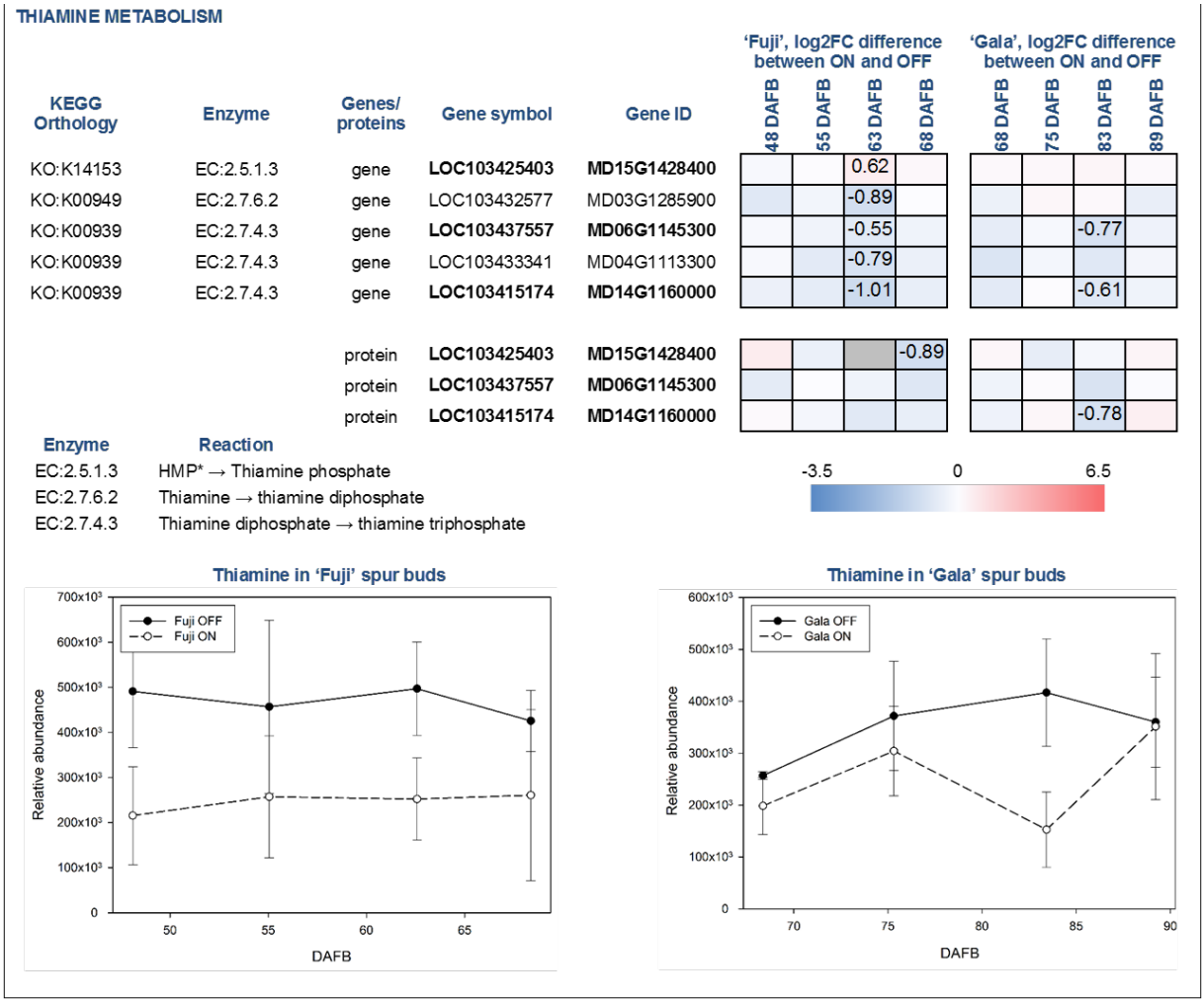


Figure 7 Relative abundance of thiamine in spur buds from 'Fuji' and 'Gala' with DEGs between ON- and OFF-trees and corresponding proteins, which are assigned to the enzymatic reactions metabolizing the compound. *HMP - 4-Amino-5-hydroxymethyl-2-methylpyrimidine diphosphate. Information about the reactions and genes assigned to thiamine metabolism was provided from KEGG library. Bold font of gene and protein IDs indicates a link between a gene and a corresponding protein illustrated in the figure. Negative log2FC values (blue) indicate a higher gene expression or higher protein abundance in OFF, positive log2FC values (red) – in ON. Cells of the same color on the heat map indicate the same level of log2FC gene (protein) expression difference between ON- and OFF-trees and do not indicate significant differences. Numbers on the heat map specify exact log2FC differences between ON- and OFF-trees and mark significant differences at multiple adjusted p value ≤ 0.05. Gray cells indicate no statistics possible due to "fill gap" function switched off (see material and methods).

Chlorogenic acid is a product of coumaric acid metabolism, where enzymes belonging to shikimate O-hydroxycinnamoyltransferases (EC:2.3.1.133) catalyze multiple reactions. Among 24 genes assigned for these enzymatic reactions in KEGG, three DEGs and one DAP were identified in our study (Figure 8). According to a gene annotation in the apple genome, all of them were classified as HXXXD-type acyl-transferase family protein and showed distinct, sometimes even inverse, expression patterns. MD13G1114800 showed up to 6.38-fold greater expression in ON-trees of both cultivars. No proteins corresponding to this gene were detected. Another gene, MD16G1108700, was higher expressed in spur buds from OFF-trees in both apple cultivars with a significant effect in 'Fuji' OFF at 55 DAFB. Its protein-product was

characterized by significantly higher abundance in OFF-trees in both cultivars two weeks prior to flower initiation. Average relative abundance of chlorogenic acid was 2.0-fold higher in ‘Fuji’ OFF-trees and 2.7-fold higher in ‘Gala’ OFF-trees compared to ON-trees with prominent cultivar differences. In ‘Gala’ ON and OFF this compound was 4.3- and 5.6-fold higher abundant than in ‘Fuji’ ON and OFF, respectively.

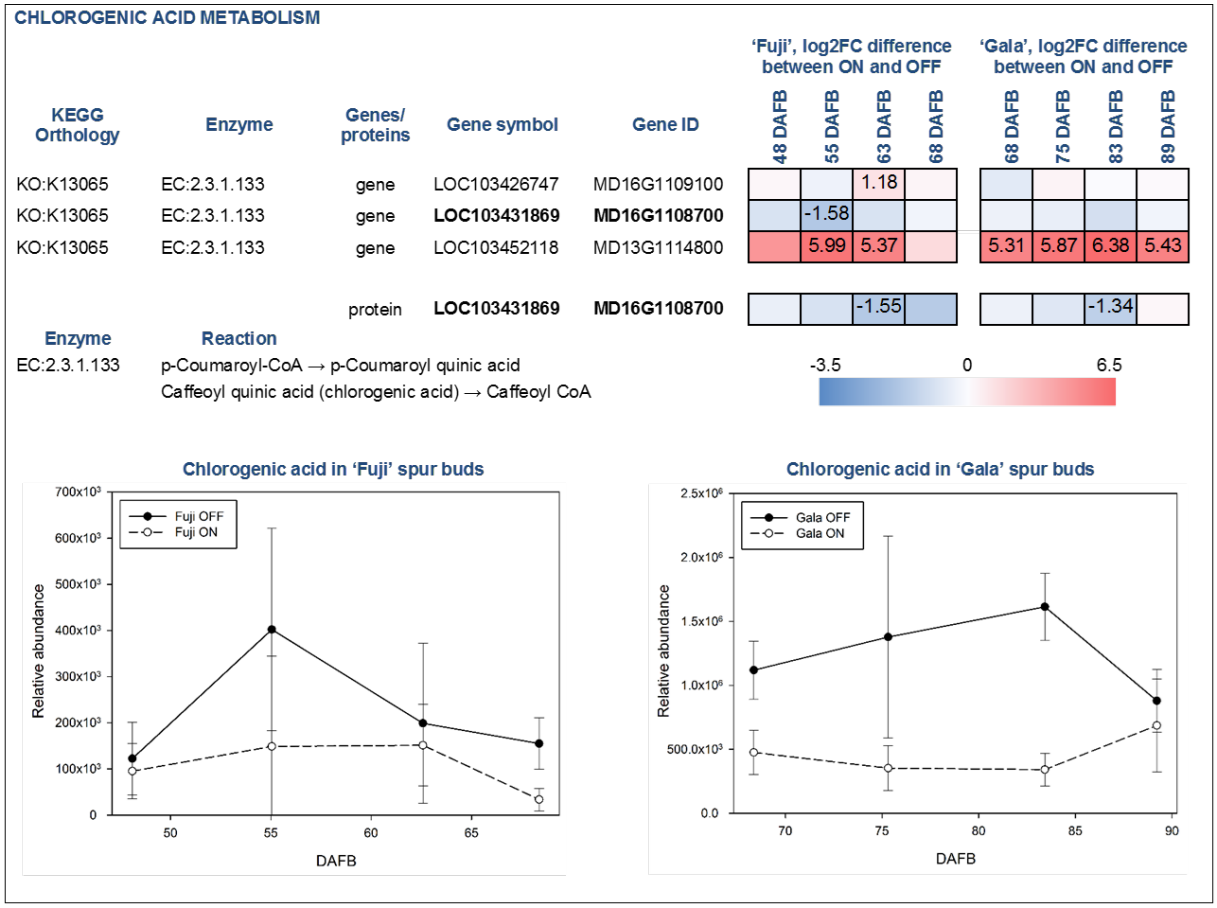


Figure 8 Relative abundance of chlorogenic acid in spur buds from ‘Fuji’ and ‘Gala’ with DEGs between ON- and OFF-trees and corresponding proteins, which are assigned to the enzymatic reactions metabolizing the compound. For detailed figure explanation see Figure 7.

Tryptophan in apple is synthesized by several reactions catalyzed by tryptophan synthase (EC:4.2.1.20), to which 5 genes were assigned using KEGG pathway analysis. However, non of them were differentially expressed between ON- and OFF-trees neither in ‘Fuji’ nor in ‘Gala’. Tryptophan detected in apple spur buds was characterized by 2.3-fold higher abundance in ‘Fuji’ ON compared to ‘Fuji’ OFF and by 2.1-fold higher abundance in ‘Gala’ ON compared to ‘Gala’ OFF. Cultivar comparison demonstrated that the relative abundance of tryptophan was much higher in ‘Fuji’ than in ‘Gala’ differing within both, ON and OFF treatments, by about an

order of magnitude (Figure 9). Furthermore, tryptophan was shown to increase in concentration towards flower initiation in ‘Fuji’ while it was more fluctuating in ‘Gala’.

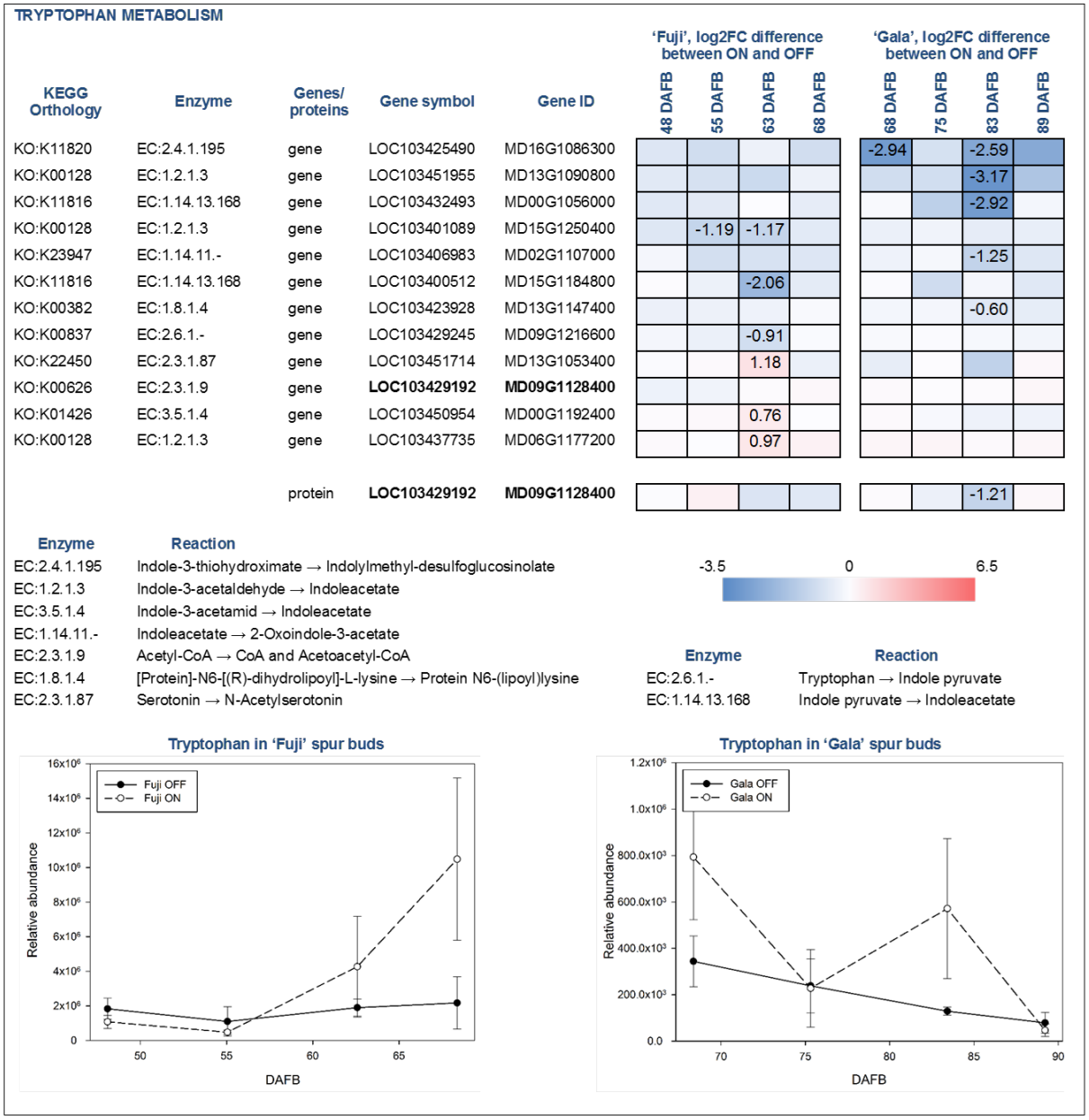


Figure 9 Relative abundance of tryptophan in spur buds from ‘Fuji’ and ‘Gala’ with DEGs between ON- and OFF-trees and corresponding proteins, which are assigned to the indoleacetate biosynthesis. For detailed figure explanation see Figure 7.

To date, 66 genes are known to be involved in tryptophan metabolism in apple. Among them, 12 were found differentially expressed between ON- and OFF-trees in any cultivar, most of which were involved in indoleacetate biosynthesis. The expression patterns of those genes were not only cultivar specific but also treatment dependent. In particular, in ‘Gala’ OFF-trees, we detected DEGs for indoleacetate biosynthesis from indole-3-acetaldehyde (MD13G1090800) and indole pyruvate (MD00G1056000), whereas no genes showed

significantly higher expression in ‘Gala’ ON-trees. In both ‘Fuji’ ON- and OFF-trees, we found DEGs coding aldehyde dehydrogenases (EC:1.2.1.3) converting indole-3-acetaldehyde into indoleacetate (MD15G1250400 in ‘Fuji’ OFF and MD06G1177200 in ‘Fuji’ ON). One gene coding indole-3-pyruvate monooxygenase (EC:1.14.13.168) that catalyzes reaction of indoleacetate biosynthesis from indole pyruvate (MD15G1184800) was higher expressed in ‘Fuji’ OFF. Another gene of amidase enzyme (EC:3.5.1.4) leading to indoleacetate by catalizing of indole-3-acetamid was found higher expressed in ‘Fuji’ ON (MD00G1192400). From the diversity of genes related to tryptophan metabolism, we could detect only one protein in the proteomic dataset (MD09G1128400). This protein was higher abundant in ‘Gala’ OFF 83 DAFB.

4.4 Discussion

Despite the fact that the apple genome has already been deciphered, even the newest high quality *de novo* genome versions still cannot provide sufficient information about promotors and repressors of flowering and to which known metabolic pathways they belong to. Many research groups attempted to identify genes that might induce flowering in higher plants; however, the majority of them used Arabidopsis as a model plant, whose ability to flower strongly depends on day length (Seo et al. 2011; Blümel et al. 2015). However, there is evidence that some homologues of the flowering regulating genes discovered in Arabidopsis also play a role in flower bud development in apple (Guitton et al. 2016). Transcriptomic analysis revealed numerous DEGs between ON- and OFF-trees two weeks prior to flower bud initiation and thereby showed that candidate genes for flower induction published in the literature is a small fraction of all transcripts differentially expressed between ON- and OFF-trees, which we could detect in this study. Gene and protein enrichment analyses illustrated metabolic pathway differences between spur buds collected from ON- and OFF-trees. In OFF-trees, we detected several enriched metabolic processes, which are assumed to lead to bud development and meristem differentiation in apple. These pathways included carbon fixation

during cell photosynthetic activity and fatty acid biosynthesis, where enzymes from pyruvate and biotin metabolism pathways are involved (Rawsthorne 2002). Fatty acids are then metabolized into different forms including glycerophospholipids, which are used to build cell membranes (Harwood 2005). Metabolic profile of apple spur buds confirmed that fatty acid structured compounds are more abundant in OFF-trees (compounds 14, 18, 19 in Figure 6). Besides that, we detected physiological pathways of amino acid biosynthesis and metabolism, metabolism of purine and pyrimidine that serve as DNA and RNA constituents, DNA replication, starch and sucrose metabolism and flavonoid biosynthesis.

Amino acid arginine, which was identified in the metabolic profiles of both apple cultivars, was significantly higher abundant in ‘Fuji’ OFF 48 and 55 DAFB in comparison to ‘Fuji’ ON (Figure 6). Besides being a protein constituent, arginine serves as a nitrogen storage in plants and enables fine-tuning of the production of nitric oxide, polyamines and potentially proline (Winter et al. 2015). However, there is so far no evidence of its involvement in flower bud development. Amino acids phenylalanine, tyrosine and tryptophan are immediate precursors for phenylpropanoid biosynthesis pathway that includes biosynthesis of flavonoids and chlorogenic acid in particular. Metabolic profiling of ‘Fuji’ and ‘Gala’ spur buds demonstrated that chlorogenic acid showed higher abundance in spur buds collected from OFF-trees. Cultivar comparisons within the same treatments showed several-fold higher abundance of chlorogenic acid in non-biennial cultivar ‘Gala’. In previous studies, chlorogenic acid was proven to inhibit IAA-oxidase and therefore to protect auxin from its inactivation (Pilet 1964). Furthermore, Lavee *et al.* (1986) reported that chlorogenic acid had auxin-like activity affecting the growth of olive shoot apices cultivated *in vitro*. We propose that chlorogenic acid might have promoting effect on bud meristem growth through the interactions with auxin metabolic pathway or might act independently having a partial hormonal activity. Transcriptome and proteome of apple buds revealed the activity of enzymatic reaction EC:2.3.1.133 that metabolizes at least 3 derivatives of p-coumaric acid including p-Coumaroyl-CoA and Caffeoyl shikimic acid, which serve as chlorogenic acid precursors. The gene MD16G1108700 and the corresponding protein assigned to this reaction were detected in apple spur buds and were

both higher expressed in OFF-trees, whereas the MD13G1114800 gene showed significantly (up to 6.38-fold) higher expression in ON-trees with no detectable protein-product. Exploring the metabolic profile of 'Fuji' and 'Gala', we revealed that one compound from the metabolic dataset with m=326.09986 and RT= 8.389 (compound 1 in Figure 6) showed similarity with a fragmentation spectrum of p-coumaric acid (peaks at 93.03, 119.05 and 163.04 m z^{-1}) suggesting that this compound and chlorogenic acid, having p-coumaric acid as a structural base, may belong to the same metabolic pathway. The unknown compound showed up to 3.55-fold higher abundance in spur buds from ON-trees compared to OFF-trees, however, no direct connection of the biosynthesis of this compound with high expression of MD13G1114800 gene could be established. None of DEGs and DAPs related to the reaction EC:1.14.14.96, that converts p-Coumaroyl quinic acid (a product of EC:2.3.1.133) to chlorogenic acid, were found in our datasets.

Another compound that was higher abundant in spur buds from OFF-trees of both cultivars was thiamine. This vitamin is frequently used in plant tissue culture and is proven to positively affect cell growth and development. It was reported that in *Plumbago indica*, *in vitro* bud formation from callus could be gained by adding the mixture of glycine, *myo*-inositol, nicotinic acid, thiamine, folic acid and biotin routinely to culture media (Nitsch and Nitsch 1967). In the multi-omics study, the thiamine biosynthesis pathway could also be detected at all three omics-levels of 'Fuji' and 'Gala' showing higher abundance in spur buds from OFF-trees that supports previous findings. Though it was impossible to conclude whether thiamine directly contributes the flower induction by influencing the bud meristem formation, it is known that thiamine diphosphate plays a role as an enzymatic cofactor in universal metabolic pathways including glycolysis and the pentose phosphate pathway. Moreover, it has the same function in mitochondrial and chloroplastic pyruvate dehydrogenases. The latter provides acetyl-coenzyme A and NADH for biosynthesis of fatty acids (Goyer 2010). KEGG library indicates that in apple, thiamine is formed by cleaving of a phosphate group from thiamine phosphate, where enzymes classified as EC:3.1.3.100 are involved. Our analyses showed no DEGs or DAPs related to this enzyme group. The gene MD15G1428400 assigned to EC:2.5.1.3,

thiamine phosphate synthase, was higher expressed in ‘Fuji’ ON at 63 DAFB, whereas the other 4 genes assigned to EC:2.7.6.2 and EC:2.7.4.3 converting thiamine into thiamine diphosphate and latter into thiamine triphosphate, respectively, were higher expressed in OFF-trees.

Among the pre-identified compounds with higher abundance in OFF-trees and thus potentially having promoting effect on flower induction, one compound showed a fragmentation spectrum similar to 1-methyladenine (peaks at 150.08, 133.05, 94.04, 82.04 and 55.03 m z^{-1}). It was characterized by up to 4.95-fold higher relative abundance in OFF-trees compared to ON-trees throughout all four sampling weeks in both apple cultivars. Notwithstanding all the efforts to classify the compound, we could not verify the proposed structure because of many differences in the fragmentation spectrum between the potential candidate and the reference spectrum of 1-methyladenine. It is well known that adenine serves as a base for the wide range of naturally occurring cytokinins (Kieber and Schaller 2014). This fact rises an assumption that the unknown compound may represent a fragment, a precursor or a product of metabolism of one of them.

Metabolic profile of ‘Fuji’ and ‘Gala’ spur buds suggests that the majority of pre-identified flavonoids were more abundant in OFF-trees (compounds 6, 8, 9, 10, 13 in Figure 6). This is in conformity with transcriptomic and proteomic profiles of spur buds collected from those cultivars, where flavonoid biosynthesis pathway was significantly enriched in OFF-trees. Metabolic profile of ‘Fuji’ and ‘Gala’ spur buds suggests that the majority of pre-identified flavonoids were more abundant in OFF-trees (compounds 6, 8, 9, 10, 13 in Figure 6). This is in conformity with transcriptomic and proteomic profiles of spur buds collected from those cultivars, where flavonoid biosynthesis pathway was significantly enriched in OFF-trees. The precise molecular masses and the fragmentation spectra of polyphenolic compounds indicated that they belong to the phloridzin and kaempferol aglycone type of flavonoids (compounds 5, 8, 10 in Figure 6) with additional C5- and C6-sugar moieties attached to the acglycones. However, the precise structure of these phloridzin and kaempferol flavonoids could not be resolved. Scientific literature provided no evidence that flavonoids may have a direct influence

on bud meristem development. However, the findings of Brown *et al.* 2001 and Peer *et al.* 2004 suggest that flavonoids play a role as negative auxin transport regulators, which would be in line with the higher abundance of flavonoids observed in OFF-trees.

In the metabolic dataset, tryptophan appeared in a cluster of small molecules, which were characterized by significantly higher abundance in spur buds from ON-trees. Moreover, cultivar comparison showed that tryptophan had 10-fold higher abundance in 'Fuji' spur buds compared to 'Gala'. These differences make this compound and the metabolic pathways, in which tryptophan is involved, interesting for further studies. Since no genes assigned for tryptophan biosynthesis differed significantly in their expression between ON- and OFF-trees, the accumulation of this compound in spur buds collected from ON-trees may be explained by suspended conversion of tryptophan to other primary or secondary metabolites. Transcriptomic analysis showed that the majority of DEGs from tryptophan metabolism pathway were assigned to indoleacetate biosynthesis, an active auxin form in plants. Metabolic profile provided no evidence on the presence of any auxin form in the analyzed apple spur buds.

Transcriptomic and proteomic analyses indicated the involvement of phytohormones in flower bud development. Based on this information, in our metabolic approach, we used reversed-phase (RP) chromatography that is known to be suitable to analyze hydrophobic compounds such as phytohormones (Pan et al. 2010). However, none of the known plant hormones could be successfully detected using the non-targeted metabolomics, most likely due to the low abundance of these compounds in apple bud tissue compared to other metabolites. Therefore, a further step towards systematic understanding of flower induction in apple would be the determination of plant hormone profiles by targeted mass spectrometry analyses using extraction protocols specifically designed for the analyses of phytohormones (Farrow and Emery 2012; Urbanová et al. 2013).

NGS and proteome analysis revealed several sugar metabolism and interconversion pathways that are actively ongoing in spur buds, which develop flower meristem, indicating that particular sugar forms might play an important role in flower bud induction and initiation. However, the

majority of MS-signals obtained from different sugar forms found in metabolic profiles of apple spur buds, could not be interpreted because the clear separation of sugar molecules with the same molecular mass using reversed-phase chromatography could not be achieved. In order to enlarge the knowledge about the involvement of simple sugar forms, such as mono-, di-, and trisaccharides in flower induction in apple, sugar analysis in apple buds is also essential. Once performed, it would help to link the activity of genes and corresponding proteins to specific sugar compounds.

The picture of flower bud induction mechanism in apple is far from complete. In the recent years, the understanding of plant flower organ formation mechanisms has strongly advanced; however, it is still unclear what the initial trigger for floral meristem formation is and how the fruit may inhibit flower induction in the adjacent spur buds. The second question may be answered by determining of which metabolites that showed higher abundance in spur buds from ON-trees are originating from the fruit. Once determined, it would narrow down the search of candidate genes, which are affected by the unknown mobile signal. In the apple metabolome, from 1,140 features with robust signals detected by non-targeted metabolomics, only 111 could be annotated with any potential compound from chemical databases, indicating that only 10% of compounds found in apple spur buds could be claimed as “knowns”. Besides the 22 compounds presented in the current work, nearly 70 hitherto unknown metabolites were found to be differentially abundant between spur buds from ON- and OFF-trees on a time-series scale.

In summary, the multi-omics approach applied for the identification of flower induction signaling molecules in apple allowed observing complex plant metabolic processes and to tracing some pathways from gene-to-product level. The data suggests that thiamine, chlorogenic acid and an adenine derivative are involved in flower bud meristem development in apple. Tryptophan was found to be more abundant in spur buds collected from ON-trees. Cultivar comparison (biennial cultivar ‘Fuji’ vs. non-biennial cultivar ‘Gala’) indicated that chlorogenic acid was 4.3-5.6-fold higher abundant in ‘Gala’ spur buds, whereas tryptophan was 10-fold higher abundant in spur buds collected from ‘Fuji’. Genes controlling tryptophan biosynthesis were not affected

by ON- and OFF-treatments, however, genes regulating tryptophan metabolism to indoleacetate showed significant expression differences between treatments and cultivars. At transcriptomic and proteomic levels, in apple spur buds collected from OFF-trees, metabolic pathways promoting tissue growth and development and thus resulting in flower bud initiation were detected. At the same time, in spur buds from ON-trees, plant hormone signal transduction pathway was enriched, suggesting its inhibiting effect on flower initiation and thus apple bud meristem fate.

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5. General Discussion

The aim of this study was to contribute to the understanding of the underlying mechanisms of biennial bearing in apple by analyzing the physiological processes in bud meristems during the time of flower bud induction. Flower induction in experimental trees was promoted or inhibited by artificially setting low and high crop loads, respectively. Using the regular bearing cultivar ‘Royal Gala’ and the biennial bearing cultivar ‘Fuji’ allowed the comparison of two slightly different developmental responses to the crop load treatments. Buds from 2-year-old spurs were sampled during the growing seasons 2015 and 2016 starting approximately four weeks after full bloom. Histological sections were prepared to (i) determine the time point of flower bud initiation which is characterized by distinct morphological changes. Then, buds that were sampled approximately two weeks before the identified flower bud initiation, were selected to analyze biochemical changes during the supposed flower induction phase. State-of-the-art multi-omics techniques, including non-targeted proteomic and metabolomic profiling and next-generation RNA sequencing, was used to (ii) identify differences in the proteomic profile of vegetative and floral buds to contribute to the identification of possible biological markers for biennial bearing behavior and (iii) to outline holistic gene-to-product pathways possibly explaining the different flowering responses to high and low crop loads.

5.1 Findings and their relevance for future work

5.1.1 The time point of bud initiation in apple

The histological analysis of bud meristems successfully identified the time point of flower bud initiation in both cultivars at the experimental site. The onset of flower bud initiation was affected by crop load, cultivar and heat accumulation. While heavy cropping delayed the onset in ‘Royal Gala’ trees for 20 days compared to ‘Royal Gala’ trees with no crop load, bud initiation in heavy cropping ‘Fuji’ trees was negligible. ‘Fuji’ trees with no crop load started initiating buds 19 days earlier than ‘Royal Gala’ trees with the same cropping status. This result served as the starting point for the subsequent biochemical analyses. The fact that the starting point of

bud initiation of two different apple cultivars under the same climatic conditions and cropping status at the same location is 19 days apart is a finding not to be underestimated for the success of future experiments. Experiments investigating flowering mechanisms in apple, must carefully consider the time factor in their experimental design. For example, in genetic studies on flowering genes, the temporal variability of flower initiation and thus the preceding flower induction can be a major source of error in the data set. However, it is not only the use of different apple varieties that can be a source of error for setting the correct time window. In the same way, it is essential to take into account the fruit set of the previous year and the number of flowers in the current year, as these factors influence the timing and also the intensity of bud initiation and can thus lead to inaccuracies when physiological or genetic studies are to be carried out specifically during the induction or initiation phase. Consequently, depending on the selection of different time points, a different question can be answered, as schematically shown in Figure 1.

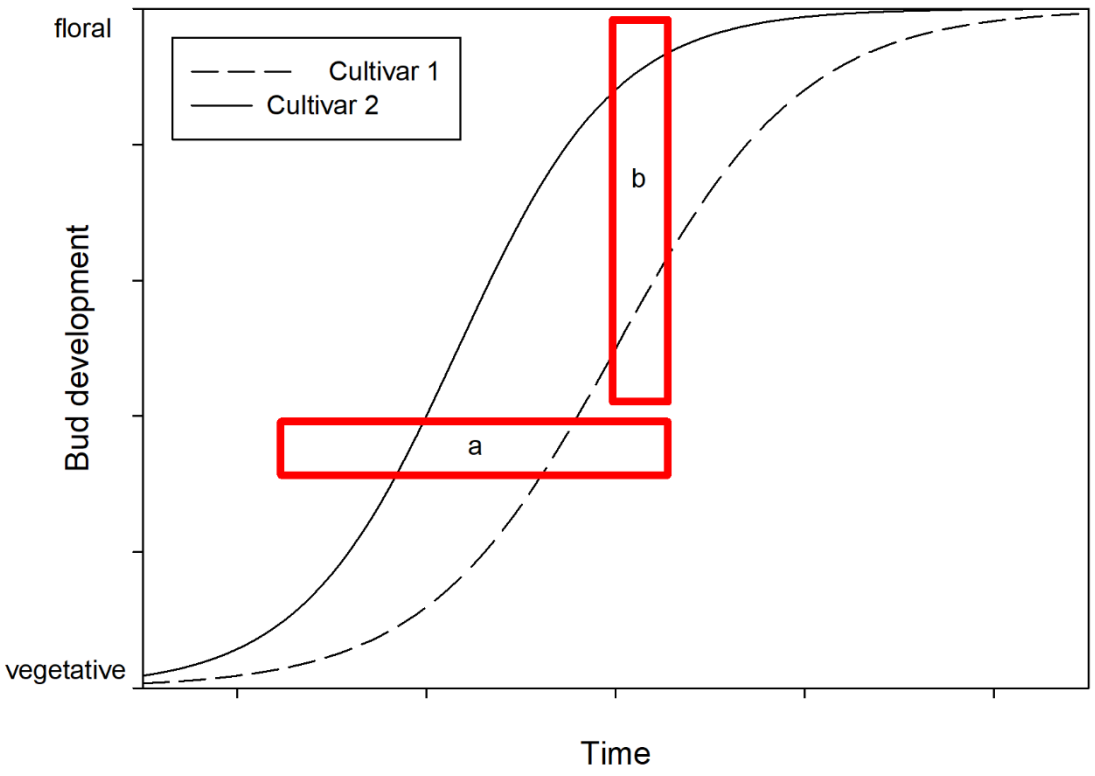

Figure 1 Schematic illustration of the importance of selecting the right samples: comparison (a) would compare buds with the same developmental stage from different time points, comparison (b) would compare buds with different developmental stages from the same time point.

For example, if one were to compare samples of two cultivars with different initiation times from the same sampling time, one would compare buds with different developmental stages. However, if one wanted to compare buds with the same developmental stage, one would have to compare different time points.

5.1.2 Proteins involved in flower bud induction: a basis for biomarker development for alternate bearing in apple

Proteomic profiling of the buds sampled during flower bud induction and during flower bud initiation revealed distinct differences in specific protein abundances depending on the cropping status. Buds from trees with a high crop load, where the flower bud initiation was inhibited and the buds primarily remained in a vegetative state, showed a decreased abundance of enzymes belonging to the phenylpropanoid and flavonoid pathways and increased abundances of histone deacetylase and ferritins. Buds growing on non-cropping trees with a high rate of flower bud initiation, showed significantly higher concentrations of proteins involved in histone and DNA methylation. These findings can serve as the basis for biomarker development that will accelerate breeding programs for apple cultivars that are less prone to alternate bearing. Thus, by introducing high-speed breeding technologies exploiting transgenic early flowering apple plants (Flachowsky et al. 2011), early screening for alternate bearing biomarkers can be performed using bud meristem tissue samples.

5.1.3 Omics data analysis: finding the needle in a haystack

The multi-omics approach showed that thiamine, chlorogenic acid, and an adenine derivative play a role in metabolic pathways promoting early flower bud development in apple, and that tryptophan was more abundant in buds collected from high-cropping trees compared to non-cropping trees.

Theoretically, it would have been possible in the present work to demonstrate the complete synthetic pathway of a metabolite. Assuming that one or more proteins synthesize a metabolite, e.g. a plant hormone, one could have also identified the underlying genes by means of the RNA-seq analysis. Thus, one could have traced complete synthetic pathways of

a metabolite that is synthesized in the bud meristem and exerts its mechanism of action locally. Alternatively, it would be plausible to identify a metabolite that originates outside the bud meristem but then triggers a signaling chain locally in the meristem, starting either at the RNA or protein level.

The fact that many differentially expressed genes were found in buds of on- and off-trees specifically at the time point two weeks before bud initiation reinforces the original assumption that the time of flower induction must probably be during this period. The serious differences in the number of differentially expressed genes and proteins between the cultivars ‘Royal Gala’ and ‘Fuji’, probably also has a trivial statistical cause. Assuming that we are trying to identify and describe a mechanism, namely that of flower induction, whose occurrence not only differs in absolute numbers between the cultivars but also takes place at different times, it is almost impossible to obtain sample material that allows a correct cultivar comparison. Since it is always a mixed sample, it must always be assumed that not only vegetative and floral buds, but also buds in different stages of development are present in the sampled bud mixture. The number of buds that have undergone floral induction two weeks before the observed floral initiation will always be greater in a strongly alternating cultivar such as ‘Fuji’ with low fruit set than, for example, in ‘Royal Gala’. This always leads to an enrichment of corresponding flower-inducing substances in the further examination of the bud material, which favors the identification of these low concentrated molecules for technical reasons alone. Thus, certain qualitative differences in the omics-profile between the cultivars do not necessarily have to have a cultivar-related origin, but may simply be due to the identification threshold of the analytical method used.

5.2 Identified difficulties and their relevance for future work

5.2.1 Prerequisites to the experimental design for interpretable results

In order to represent environmental conditions as realistically as possible, a field trial was chosen for the present work. In a perfect experimental design, the factors that have the most

influence on the physiology and therefore the treatment response of the tree would have been similar across all experimental trees, or completely randomized across all experimental trees if there are differences due to their inherent nature. This would include the apple cultivar to rule out any interactions between cultivar and location in the experimental field. However, it was necessary to work with trees that had already completed the juvenile phase and have a similar age in order to observe normal flowering behavior. Thus, recourse had to be made to trees that had already been planted. In the available experimental orchard, the apple cultivars used, 'Royal Gala' and 'Fuji Raku Raku', were planted in rows, i.e. not randomized. However, the following consideration justifies this apparent weakness of the experimental design: it was not reasonable to assume that a possible interaction effect between cultivar and location would exceed the treatment effect. Consequently, it was more important to randomize the experimental treatment rather than the cultivar. It should also be mentioned again how important it is to have a sufficiently large number of trees available. For the different analyses of the present work, a large number of buds was needed. Thus, 55 buds had to be collected per tree, time point and replicate resulting in more than 20,000 sampled buds to obtain sufficient material. However, to prevent an effect of the bud collection on the physiology of the tree, a tree could only be sampled once. This was only possible by sampling a different, randomly selected tree at each time point. Thus, with four replicates per date, a new set of four trees had to be randomly selected each time. This sampling strategy also simplified the statistical model, as it excluded any effect of the previous sampling on the current sampling.

5.2.2 Collecting the right sample at the right time

Proper bud sampling turned out to be more complicated than first thought. Several factors are essential to reliably collect the correct sample material. Since bud initiation is not 100% synchronous within a tree, it must always be considered that buds are at different stages of development on the tree at a given time. Externally, it is not recognizable to the buds, especially in the early stages of development, whether they are vegetative or floral buds. To clearly determine the stage of development of the bud, it is necessary to make histological

sections. However, this process completely destroys the bud, which makes further analysis impossible.

Consequently, a bulk sample of buds collected from a given tree at a given time is always a mixture of vegetative and floral buds. Therefore, when creating the experimental design, it is necessary to define exactly what kind of buds are needed to answer the research question. If, as in the present work, analytical methods are used that require a certain minimum amount of dry material, it is necessary to process a composite sample to obtain the minimum amount. Consequently, care must be taken to obtain bud mixtures that are highly likely to correspond mostly to the bud stage that one wishes to analyze. For this, the foundation must be laid with the help of the experimental design: experimental treatments must be chosen that aim to elicit the most extreme physiological responses possible. Since vegetative and floral buds were studied in the present work, two experimental treatments were chosen trying to elicit exactly the opposite developmental pathways of the buds. Complete flower thinning at full bloom on the one hand and maintaining the high number of flowers without any thinning on the other hand, achieved, at least statistically, the best possible result.

5.2.3 Somewhere in the middle: correct classification of bud developmental stages

In preparation for the histological examination of the buds, several test sections were made to understand how reliably the center of the bud meristem could be determined. For the histological study, 100 sections per bud had to be made to locate exactly that part of the meristem that met the previously defined criteria to select the section which serves as the reference for classifying the developmental stage. Without the large number of tissue sections, the center of the meristem would have been misinterpreted several times and the buds would have been classified into an incorrect stage of development. The preparation of a total of approximately 30.000 histological sections for the present study increases the power of the data and suggests that similar histological studies with fewer sections are not comparable in their statistical robustness. Consequently, the omission of information on the exact histological

methodology and the number of sections made per bud in the relevant scientific work is critical (McArtney et al. 2001; Foster et al. 2003; Oukabli et al. 2003; Hoover et al. 2004).

5.2.4 Limits of detection: transcription factors and phytohormones

A non-targeted approach was deliberately used in this work to obtain a picture as comprehensive as possible of the molecules involved in flower induction. Especially for the part of the proteome analyses, this has the advantage of finding the broadest possible correlations and protein synthesis pathways. The size of the proteins included in the statistical analysis after several filtering steps averaged 52.16 kDa, with a maximum of 674.78 kDa and a minimum of 4.61 kDa as shown in Figure 2.

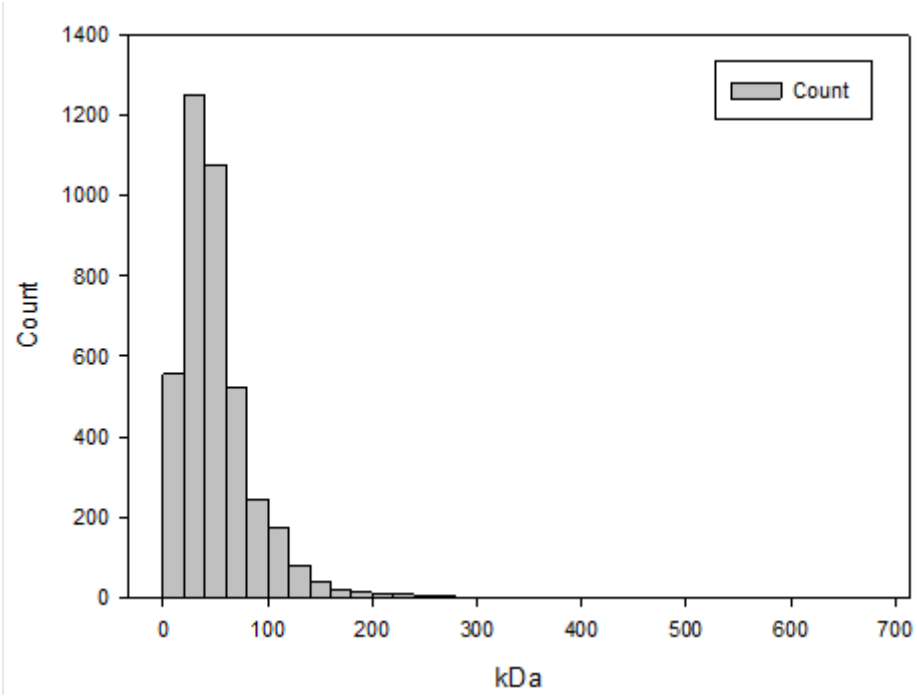

Figure 2 Histogram showing the molecular mass distribution in kDa of the 4020 proteins that were used for the proteomic analyses of the current work.

The fact that the proteomic analyses could not quantify transcription factors in sufficient quantities to evaluate them statistically correct may suggest that the molecules were either too small or present in too low concentrations in the sample material. The fact that the TFL-1 protein, a possible floral repressor, has a mass of approximately 19.3 kDa indicates that the protein could have been identified by the method. And indeed, the protein was found with relative certainty in one of the 100 samples: on July 14, 2015, i.e. 75 dafb, the TFL-1 protein

could be identified in a single replicate of the cultivar 'Royal Gala' from trees with the 'Off' treatment. However, the finding in a single sample, with only two peptides of which only one was unique, is not sufficient to make statistically robust evaluations. It can be concluded that transcription factors are not necessarily too small molecules to be found by the mass spectrometry method used, rather their concentration in the tissue is much too low, causing identification and relative quantification to fail too often at the detection threshold. It is possible that future experiments with marker-assisted proteomic analyses using isobaric tags for relative and absolute quantitation (iTRAQ), where it is possible to pool 4 or even 8 samples with different conditions, might be able to quantify such proteins with low concentrations.

The complexity of analyzing small biomolecules at low concentrations, has also been demonstrated in the metabolomics analysis. For a statistically robust evaluation and interpretation of the results, great importance was attached to the unambiguous identification of the metabolites. Not only the detailed comparison of the fragmentation spectra with the relevant databases, but only the comparison with spectra of synthetic reference compounds obtained with the same instrument and the same experimental protocol allowed the reliable identification of the metabolites. A similarly meticulous metabolomics approach has not been reported for apple bud omics analyses before. The fact that only 111 of the 1140 unique features could be assigned to known compounds, of which only five could be confirmed beyond doubt with reference compounds, shows that metabolome analyses are still highly complex and that a great deal of detailed work is required to obtain usable and interpretable results. It also turned out that the non-targeted approach for the metabolome analysis was not able to identify and quantify plant hormones. According to the literature, these important signaling molecules most likely play essential roles in the flower induction process (Nitsch and Nitsch 1967). How exactly plant hormones are to be classified within the flowering mechanism or also within the different bearing behavior of the varieties ‘Fuji’ and ‘Royal Gala’ could not be clarified in the present work. For this purpose, further targeted mass spectrometer analyses are necessary, using extraction protocols specifically developed for the analysis of plant hormones.

6. Outlook

To explain the complex physiological processes involved in flower development, the scientific literature makes use of various models (Hanke et al. 2007). In short, there are floral repressor genes such as TFL1 and floral promoters such as MdFT1 and MdFT2 (Haberman et al. 2016). However, to date there is no complete flowering model for apple that explains the complete flower development starting with flower induction in the first year of the two-year reproductive cycle. In particular, the physiological basis of alternate bearing and the within-tree communication necessary for it still lack knowledge of the involved signaling molecules.

The present work could not paint a holistic, detailed picture of a signaling pathway that could fully explain the influence of this year's fruiting on the intensity of flower formation in the following year. Ideally, a protein or a metabolite would have been found that was present in different concentrations depending on fruit set and cultivar. This signal molecule could have had a reinforcing or inhibiting influence on flower induction, i.e. the irreversible initiation of the floral development path of a bud that until then had followed the vegetative development path. The origin of the molecule could have been, for example, young developing fruits, especially their seeds as it already has been assumed in literature (Marino and Greene 1981). The site of action, on the other hand, would have to be the developing meristem of adjacent buds. In the meristematic tissue, if it had an inhibitory effect on flower induction, reactions would then have to occur that would allow the floral developmental pathway suppressive mechanisms to continue to operate.

The selection of proteins, metabolites, and genes that the current work produced through its broad, non-targeted approach provides a comprehensive data base for future, more targeted analyses. The publicly deposited data allow easy and effective screening of temporal expression and relative concentration patterns of many different molecules that research groups can screen for candidate suitability in future studies. In addition, PCR analyses, for example, can be used to specifically screen and validate the genes of various candidate proteins in follow-up experiments.

The field experiment could show that the accumulated heat sum has an influence on bud development. However, it could not be conclusively clarified whether the delaying influence of heavy fruiting is influenced by the heat sum or not. Trees with strong fruiting have been shown to start bud initiation later than trees with weak fruiting. However, the rate of bud development was not affected. Now, one might consider what would happen if the growing season were extended, i.e., if the tree could compensate for the delayed start of the bud development by delaying the onset of paradormancy. Such a hypothesis could be tested in climate chambers with controlled growth conditions.

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Affidavit

pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic

   Physiological, metabolic and molecular basis of biennial bearing in apple

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

Stuttgart, 30.10.2022 
(Place, date) (Signature)

Affidavit
Information

The University of Hohenheim requires an affidavit declaring that the academic work was done independently in order to credibly claim that the doctoral candidate independently completed the academic work.

Because the legislative authorities place particular importance on affidavits, and because affidavits can have serious consequences, the legislative authorities have placed criminal penalties on the issuance of a false affidavit. In the case of wilful (that is, with the knowledge of the person issuing the affidavit) issuance of a false affidavit, the criminal penalty includes a term of imprisonment for up to three years or a fine.

A negligent issuance (that is, an issuance although you should have known that the affidavit was false) is punishable by a term of imprisonment for up to one year or a fine.

The respective regulations can be found in Sec. 156 StGB (Criminal Code) (false affidavit) and in Sec. 161 StGB (negligent false oath, negligent false affidavit).

Sec. 156 StGB: False Affidavit

Issuing a false affidavit to an authority body responsible for accepting affidavits or perjury under reference to such an affidavit shall be punishable with a term of imprisonment up to three years or with a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Affidavit:

Subsection 1: If one of the actions described in Secs. 154 and 156 is done negligently, the action shall be punishable by a term of imprisonment of up to one year or a fine.
Subsection 2: Impunity shall apply if the perpetrator corrects the false information in a timely manner. The regulations in Sec. 158 (2) and (3) apply mutatis mutandis.

The German original version of this affidavit is solely valid; all other versions are merely informative.

I have taken note of the information on the affidavit.

Stuttgart, 30.10.2022 
(Place, date) (Signature)

Curriculum Vitae

Julian Andreas Kofler / Stuttgart, 30.10.2022 

Mendelssohnstraße 24, 70619 Stuttgart
Geboren 18.06.1989 in Leonberg

Schulische Ausbildung

1995-1999	Grund- und Hauptschule, Höfingen
1999-2008	Johannes-Kepler-Gymnasium Leonberg, Abitur

Zivildienst

2008-2009	Karl-Georg-Haldenwang-Schule, Leonberg

Studium

2009-2012	Universität Hohenheim Bachelor of Science „Nachwachsende Rohstoffe und Bioenergie“ 2-monatiger Forschungsaufenthalt in Vietnam
2012-2015	Universität Hohenheim Master of Science „Agricultural Science in the Tropics and Subtropics“ 4-monatiger Forschungsaufenthalt in Ghana
2015-2023	Universität Hohenheim Promotion Agrarwissenschaften „Physiological, metabolic and molecular basis of biennial bearing in apple”

Berufserfahrung

April 2005	Praktikum beim GENO-Verband Stuttgart im Bereich Informationstechnologie/Organisation
März/April 2011	KWS Maiszuchtstation Gondelsheim, Mitarbeit in der Aufbereitung von Leistungsprüfungen
August/Oktober 2011	Praktikum im Sonderforschungsbereich 564 der Deutschen Forschungsgemeinschaft „Nachhaltige Landnutzung und ländliche Entwicklung in Bergregionen Südostasiens“ in Vietnam
2010-2012	Wissenschaftliche Hilfskraft am Institut für Pflanzenproduktion und Agrarökologie in den Tropen und Subtropen, Universität Hohenheim
2013-2014	Wissenschaftliche Hilfskraft am Institut für Kulturpflanzenwissenschaften, Universität Hohenheim
2015-2021	Geschäftsführer Bereich Vertrieb und Marketing Intertrop GmbH, Stuttgart
2021-heute	Account Manager communicationAds GmbH & Co. KG, Nürnberg