Hort Innovation

Final Report

Physiological, metabolic and molecular basis of biennial bearing in apple

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Physiological, metabolic and molecular basis of biennial bearing in apple (AP15002)

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Content

Summary

Flower bud induction in fruit trees is affected by biennial bearing, which is characterized by alternating years with high (ON) and low (OFF) crop loads, respectively. Such cropping behavior is not observed in all horticultural crops and each cultivar within a given species, indicating that the genetic and physiological mechanism of biennial bearing must be conserved. In apple (Malus ×domestica Bork.), biennial bearing is differentially expressed among the genetic diversity of commercial cultivars. OFF-years typically result from a poor flower bud formation process due to a high crop load in the previous year, suggesting that the fruit inhibits the concomitant development of the adjacent spur bud. The overall research goal of the current project was to reveal the largely unknown physiological and molecular mechanisms of biennial bearing in apple, thereby to better understand the underlying pathways and triggers of flower induction that might facilitate intervention opportunities for controlling apple crop load and thus ensuring stable apple production. Consequently, we used new plant research technologies to reveal the presence and quantity of differentially expressed genes (transcriptomics), proteins (proteomics) and small-molecule chemicals as for example sugars, amino acids, flavonoids or lipids (metabolomics) in apple bud sample at a given time. Moreover, functional genomics then described gene, protein and metabolite functions and interactions. The large-scale omics-studies was applied to apple spur buds from the strongly biennial bearing cultivar 'Fuji', clone 'Raku-Raku', and the regular bearing cultivar 'Royal Gala', clone 'Galaxy'. Spur buds were sampled at weekly intervals for up to 15 weeks after full bloom in the growing seasons 2015 and 2016 from trees of both cultivars that were adjusted to ON and OFF cropping levels to establish heavy and no fruit loads, respectively.

Histological studies determined whether a bud remained vegetative or exactly when it committed to flower formation. Consequently, a time series of bud microscopy analysis, the onset of structural changes within the bud tissue and thus the time of transition from a vegetative to floral bud was defined. Samples collected prior to this time point were then used for the omics-studies, since it is of particular interest to determine any developmental changes that lead to flower formation. Logistic regression modelling of histological data predicted the onset of flower bud initiation in 'Fuji' and 'Gala' and revealed a strong relationship between flower initiation and crop load as well as between flower initiation and growing degree day temperature accumulation. Specifically, the onset of flower bud formation in heavy cropping 'Gala' trees was delayed for 21 days compared to trees with no crop load, but the rate of initiation was not affected by crop load. In contrast, flower bud formation on heavy cropping 'Fuji' trees was minor, whereas non-cropping trees started to initiate flower buds 18 days earlier than non-cropping 'Gala' trees. The results indicate, that the genetic make-up of the cultivar determines the onset of the flower bud formation; however, it can be delayed by increasing crop loads and low temperatures.

Next generation RNA sequencing of 'Fuji' and 'Gala' spur buds revealed 40916 genes (out of 52741 total genes) that were successfully mapped to the double haploid 'Golden Delicious' genome. After statistical data processing, 6967 genes in 'Fuji' and 3426 genes in 'Gala' showed significant differences in their expression levels between ON- and OFF-trees in any of the four selected sampling weeks, respectively. Moreover, the data suggest that developmental processes of spur buds collected from 'Fuji' ON-trees were strongly suppressed whereas spur buds from 'Fuji' OFF-trees were reaching the flower initiation stage. Many differentially expressed genes were associated with proteins and metabolites involved in plant hormone signaling, sugar metabolism and transcriptional regulation, suggesting that the presence of fruit alters the metabolic processes of the adjacent spur bud by holding it in a vegetative "standby" mode.

A total of 225 proteins were found to be differentially expressed between buds from ON and OFF-trees. Downstream analysis revealed that 35 proteins had also the corresponding gene significantly expressed. Further analysis of those proteins suggested an involvement of the aromatic amino acids (AAA) phenylalanine, tyrosine and tryptophan in developing buds from OFF-trees. These essential AAAs are building blocks for proteins and serve as precursors of several secondary metabolites, including auxins and phenylpropanoids. The latter are catabolized to a wide range of compounds, including chlorogenic acid. Indeed, metabolic profiling indicated that the same metabolites were found in 'Gala' and 'Fuji' bud samples; however, the abundance of each metabolite varied depending on the cultivar and the time of sampling. More specifically, cytokinins, chlorogenic acid and thiamine were more abundant in OFF than in ON-trees and these compounds were previously described to increase the rate of flower bud formation. In contrast, the results further indicate that auxins, which are synthesized in fruit, move to the adjacent spur buds and suppress flower bud development. Moreover, the apple cultivars differed in auxin biosynthesis in the fruit that may explain the cultivar dependent degree of biennial bearing.

Keywords

apple, *Malus ×domestica* Borkh., biennial bearing, flower induction, spur buds, histological sectioning, proteomics, metabolomics, transcriptomics, phytohormones, multi-omics

Introduction

Flower induction, initiation and differentiation are developmental stages that vegetative apple spur buds need to undergo on their developmental pathway to become floral. Flower induction is commonly defined as a point in time when a vegetative bud meristem perceives an unknown signal to develop new tissue structures to form a flower meristem. In contrast, flower initiation is characterized by distinct morphological meristematic changes (Foster *et al.* 2003; Hanke *et al.* 2007). While flower initiation can be determined by bud histological sectioning, the identification of the exact time of flower induction still remains unknown. Based on the accumulated knowledge to date, some authors proposed hypothetical models of flower induction (Xing *et al.* 2015; Zuo *et al.* 2018); however, none of them could fully explain the molecular and physiological background of this developmental process. Understanding of the flower induction mechanisms is of a great importance for controlling apple crop load, inducing a stable cropping behavior over years and thus alleviating the production limitation caused by biennial bearing.

The term biennial (or alternate) bearing of horticultural crops describes erratic yields with a crop load in the following year strongly depending on the crop load of the previous year. Biennial bearing is frequently triggered by adverse environmental conditions, such as spring frosts, resulting in an OFF-year with low yield, subsequently an ON-year with high yield of small-sized fruit before returning to the OFF cropping status and starting the new repetitive cycle (Wünsche and Ferguson 2005). Biennial bearing in apple induces also variable yearly yields and growers usually remove excess flowers and fruitlets on ON-trees with heavy fruit loads to increase fruit size in the current season and the amount of bloom in the subsequent season. This moderately effective horticultural tool is extremely time-consuming and costly, but yields can still be considerably reduced. The value of the apple industry would increase if more consistent crop loads could be achieved for those apple varieties that are characterized by ON- or OFF-years.

It is estimated that about 30 % of the worldwide available commercial apple cultivars are susceptible to some level of biennial bearing. Considering that the annual value of the Australian apple production is over 650 million AUD, alleviating the ramifications associated with biennial bearing would likely lead to an increase in total industry revenue of about 50-65 million AUD. Kevin Sanders, orchardist and project partner in the Yarra Valley, agrees: “We lose 20-30% of our income every year due to biennial bearing. The ability to even out biennial bearing would be extremely valuable and assist with industry wide planning”.

The reduced flower induction rate on high yielding apple trees was frequently explained by sink-source interactions between fruit and buds developing concurrently within the growing season, with fruit being a stronger sink for carbohydrates compared to buds (Lenz 1979). Numerous *in vitro* experiments confirmed the importance of carbohydrate supply for bud flower induction. 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 further found that plant hormones and hormone-like acting compounds are strongly involved in the flower bud induction process. In *Plumbago indica*, *in vitro* bud formation from callus tissue was achieved in the presence of cytokinins and adenine and was 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). With the appearance of gene detection techniques, considerable research was devoted to the discovery of genes, 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 considerable advancements in multi-omics approaches, the current knowledge about the proteome and metabolome of apple is very limited and so far is only described 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 metabolomic data sets of apple buds in relation to biennial bearing are still missing. To study the underlying biological processes involved

in flower induction and to trace them from gene to product, it is necessary to combine several omic-technologies in an attempt to better understand the interplay between genes, proteins and metabolites determining the reproductive development of plants.

In this project, we used a holistic multi-omics approach (large-scale analyses of gene expression, proteins and metabolites), targeting the flower induction mechanisms of the biennial bearing apple cultivar 'Fuji' and of the regular bearing apple cultivar 'Gala'. Apple spur buds from ON- and OFF-trees, respectively, were collected weekly from 30 days after full bloom throughout the growing seasons of 2015 and 2016. Histological sectioning of apple buds allowed identifying the flower initiation time points for 'Fuji' and 'Gala'. RNA, proteins and metabolites extracted from buds over four weeks leading up to flower initiation, covering the assumed period of flower induction, were analyzed using RNA-sequencing technology and electrospray ionization mass-spectrometry. This approach created multi-omics profiles of apple spur buds in an attempt to reveal differences between ON- and OFF-trees.

The goal of this research project was to reveal the largely unknown physiological and molecular mechanisms of biennial bearing and thereby to understand why apple does not develop sufficient number of flowers following a year with a high crop load. The results will lead to more wealth of knowledge of the physiological pathways involved in flower development and hence to more effective crop management practices that reduce biennial bearing in susceptible apple cultivars. Moreover, the project outcome will be utilized to induce regular and uniform flowering by manipulating gene expression levels and to identify potential molecular markers for targeted selection of genotypes without the trait 'biennial bearing' in breeding populations.

Methodology

Experiments at the Centre of Competence for Fruit Cultivation

The trial included 130 trees on M9 rootstock of the apple cultivars 'Fuji', with a strongly biennial behavior, and 'Royal Gala' (for convenience, the cultivar name 'Gala' is used in this report) with a non-biennial growth habit, respectively. Half of the trees from each cultivar were completely thinned (Figure 1a, b) by hand at full bloom to establish OFF-trees that carried no fruit, whereas the other half of the trees were not thinned and maintained as ON-trees a natural high crop load.

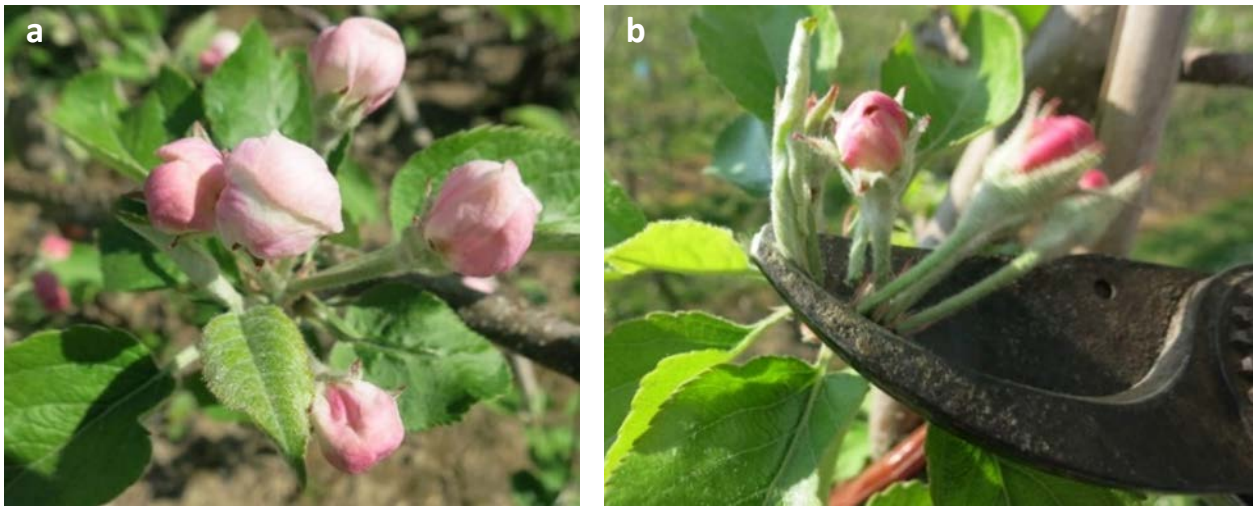


Figure 1. Apple flower clusters of 'Gala' (a) and flower thinning by using secateurs (b).

Trees were randomly assigned to both treatments and spur buds from 2-year-old wood were sampled over 15 weeks in 2015 and 9 weeks in 2016, respectively. Starting 30 days after full bloom, a new set four randomly selected trees were used at each sampling date for ON- and OFF-trees of both cultivars, respectively. A total number of 57 (in 2015) or 47 (in 2016) spur buds (Figure 2a, b) were collected from each tree at each time point: 2 buds for histological sectioning, 5 buds for RNA sequencing and gene expression analysis and the remaining buds for proteomic and metabolic profiling (Figure 3). Spur leaves and brown-colored scales, surrounding the bud, were removed (Figure 2c, d) to facilitate histological analysis and to prevent phenolic contamination during protein and nucleic acid extraction. The apical bud meristem was then isolated using a sharp scalpel.

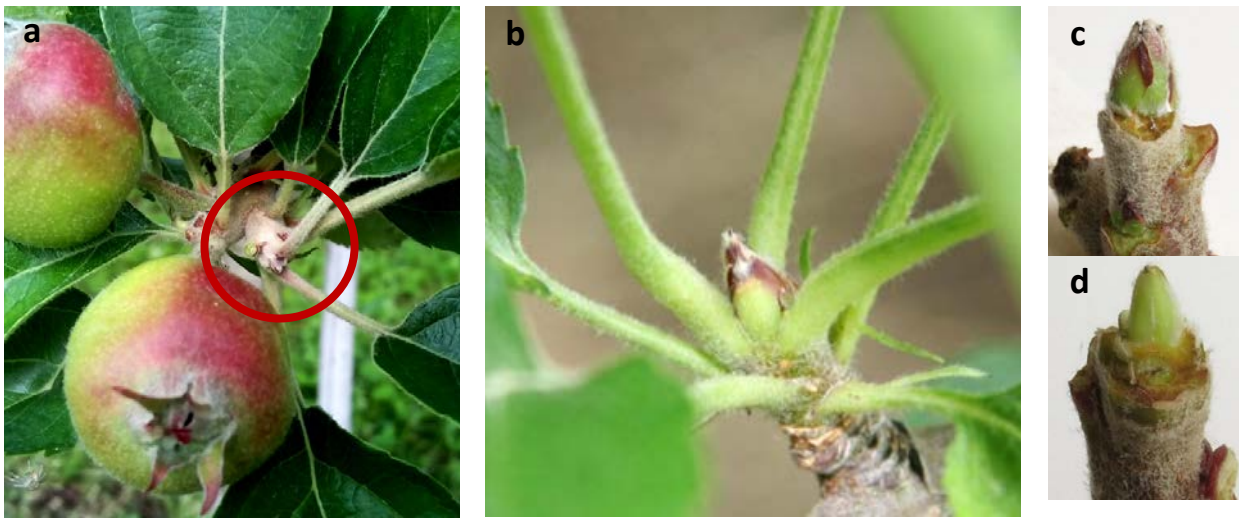


Figure 2. Buds growing on 2-year-old spur wood (a, b) and after removal of leaves (c) and scales (d) were used for various tissue analyses.

Specimens for histology were fixed in a 3.7% formaldehyde, 5% acetic acid and 50% ethanol solution, stored at 5°C, whereas all the other bud samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Specimens from each biological replicate were kept in separate safe-lock tubes.

The large-scale analyses of gene expression, proteins and metabolites (multi-omics) is a systematic study of the unique chemical fingerprint of a given plant tissue at specific developmental stage. In the growing seasons of 2015 and 2016, approximately 20 000 buds were collected for histology, gene expression, proteomics and metabolomics. Figure 3 displays the experimental design in 2015 with 15 sampling dates from 28 May to 2 September and respectively in 2016 with 8 sampling dates from 15 June to 3 August and 1 sampling date on 31 August. Figure 4 shows the tree to sample analysis workflow of the experiments in both years.

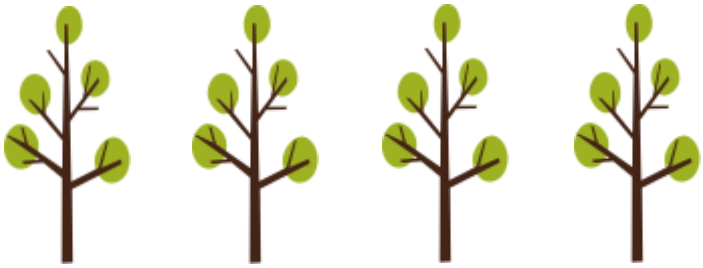
Cultivars	Treatments	Rep 1	Rep 2	Rep 3	Rep 4
'Gala', 'Fuji'	ON, OFF				
2015	RNA	5	5	5	5
2015	Proteome/Metabolome	50	50	50	50
2015	Histology	2	2	2	2
2016	RNA	5	5	5	5
2016	Proteome/Metabolome	40	40	40	40
2016	Histology	2	2	2	2

Figure 3. Experimental design in 2015 with 15 dates (28/05-02/09 2015) and in 2016 with 8 dates (15/06-03/08 2016) and 1 date at 31/08 2016).

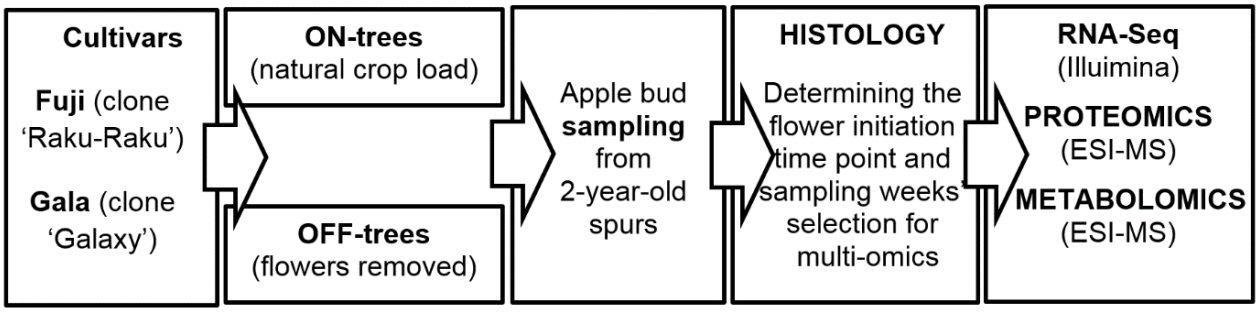


Figure 4. Workflow of the experiment.

Experiments at the Research Orchard of the University of Hohenheim

In the growing season 2015 and 2016, field experiments were carried out with the apetalous parthenocarpic apple cultivar 'Spencer Seedless' and the commercial cultivar 'Kanzi' ('Nicoter'). In both years, one hundred 4 respectively 5-year-old 'Spencer Seedless' trees growing on M9 rootstocks were used. Half of the trees were hand-pollinated, using a pollen mixture collected from different apple cultivars. The other half of the trees served as untreated controls and were not pollinated. Throughout bloom in mid-May, the king and first lateral flowers of each flower cluster (Figure 5a) of each selected tree were pollinated (Figure 5b) and all remaining flowers thinned by hand.

In 2015, a new set of four randomly selected single trees of each treatment was sampled weekly from 4 to 16 weeks after full bloom, respectively. Samples from each tree included eight fruit and eight subtending buds on 2-year-old spur wood, amounting to a total of 832 buds and fruit, respectively (Figure 6). In 2015, 5-year-old 'Kanzi' trees grown on M9 rootstock were used as an additional positive seeded control. Eight spur buds and eight fruit were collected from a new set of four randomly selected trees at each time when samples from 'Spencer Seedless' were taken, giving a total of 416 buds and fruit, respectively (Figure 6). In 2016, a new set of three randomly selected 2-tree plots of each treatment was sampled from 4 to 12 weeks after full bloom, respectively. Thirty spur buds from each plot and, assuming that 85% of the pollinated flowers developed into seeded fruit, 100 seeded fruit and 50 parthenocarpic, non-seeded fruit were collected at each sampling date (Figure 7). Consequently, a total of 1620 buds and 1350 fruit were collected throughout the experiment.

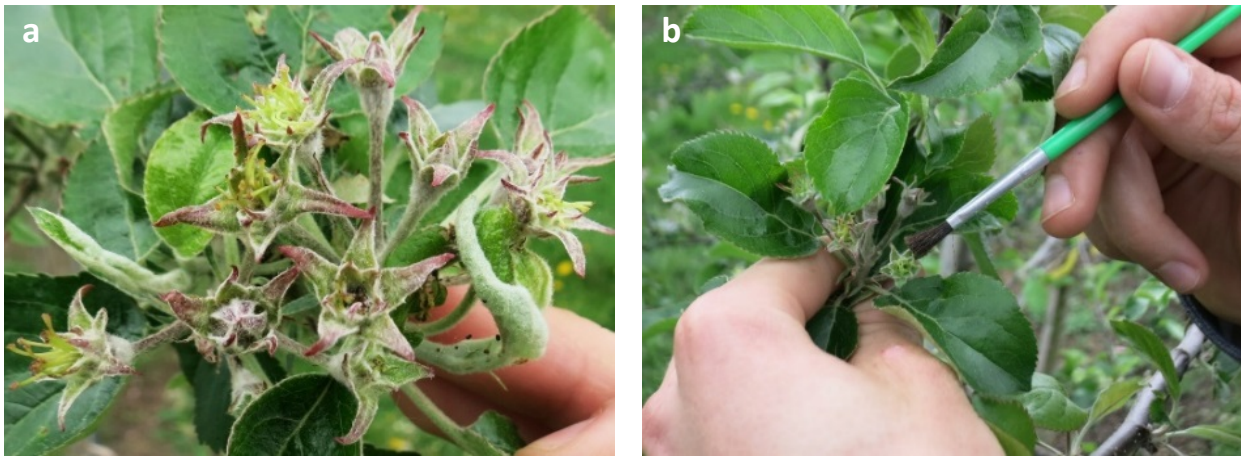


Figure 5. Flower clusters of 'Spencer Seedless' during full-bloom in May (a) and hand-pollination (b).

Immediately after harvest, fruit were placed with their peduncles on 24-well plates (Figure 8a) with each well containing 2.5 mm of buffer solution (0.1 M phosphate buffer at pH of 6.2 and solidified with 0.8% agar). To collect phloem-derived proteins, metabolites and plant hormones, 200 mM ethylenediaminetetraacetate (EDTA) was added to prevent phloem blockage. Mobile signals diffused into the buffer solution for 20h at 20°C and after incubation, plates were stored at -20°C until analysis.

Maximum diameter and weight of all collected fruit were also determined before they were cut through the equatorial plane to count and collect seeds in the carpellary tissue. Flesh tissue from the mesocarp (Figure 8b) was sampled and stored in polypropylene containers at -20°C until analysis for metabolites and proteins.

Cultivars	Treatments	Rep 1	Rep 2	Rep 3	Rep 4
'Spencer Seedling'	parthenocarp pollinated				
'Kanzi'	none				
'Spencer'/'Kanzi	RNA	8	8	8	8
'Spencer'/'Kanzi'	Diffusates IAA	4	4	4	4
'Spencer'/'Kanzi'	Diffusates GA	4	4	4	4
'Spencer'	Fruit flesh	2 x (4 fruit)	2 x (4 fruit)	2 x (4 fruit)	2 x (4 fruit)
'Spencer'	Seeds	2 x (4 fruit)	2 x (4 fruit)	2 x (4 fruit)	2 x (4 fruit)

Figure 6. Experimental design in 2015 with 13 sampling dates from 9/6 to 3/9 2015).

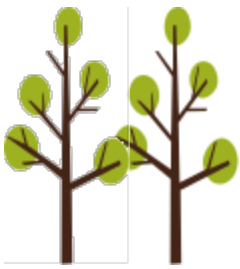
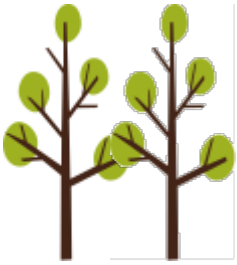
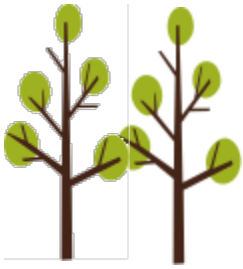
Cultivar	Treatments	Rep 1	Rep 2	Rep 3
'Spencer Seedling'	parthenocarp pollinated			
RNA		(3 + 3)	(3 + 3)	(3 + 3)
Proteome/ Metabolome		(11 + 11)	(11 + 11)	(11 + 11)
Histology		(1 + 1)	(1 + 1)	(1 + 1)
Diffusates parthenocarp IAA			(25 from 6 trees)	
Diffusates parthenocarp GA			(25 from 6 trees)	
Diffusates pollinated IAA			(50 from 6 trees)	
Diffusates pollinated GA			(50 from 6 trees)	
Fruit flesh		(4 + 4 fruit)	(4 + 4 fruit)	(4 + 4 fruit)
Seeds		separately from up to 100 pollinated fruit		

Figure 7. Experimental design in 2016 with 9 sampling dates from 20/6 to 8/8 2016 and at 29/8 2016.

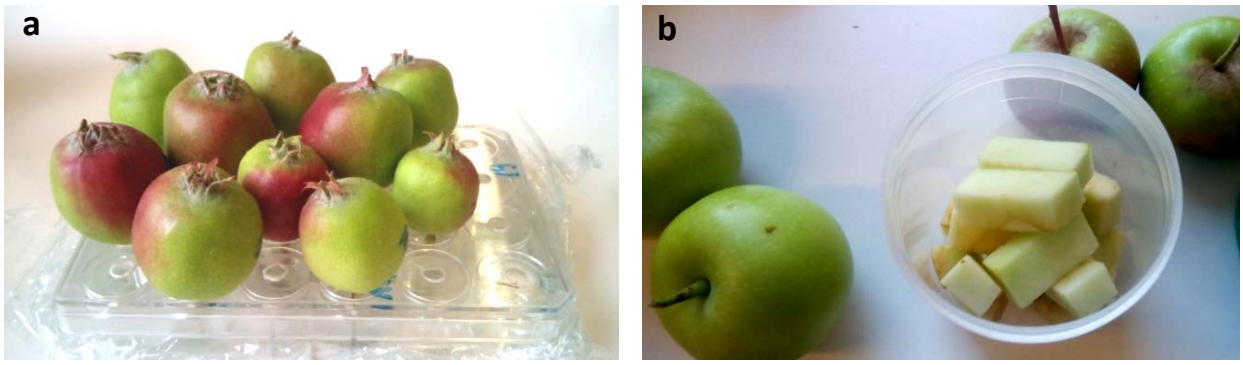


Figure 8. 'Spencer Seedless' apples on a diffusate plate (a), fruit flesh samples before freezing (b).

Multi-omics analysis (Next Generation RNA sequencing, proteomic and metabolic profiling)

Flower bud induction, initiation and differentiation is a complex phenomenon, including numerous pathways and mechanisms at genetic, proteomic and metabolic levels. The approach uses new plant research technologies to reveal the presence and quantity of differentially expressed genes (transcriptomics), proteins (proteomics) and small-molecule chemicals as for example sugars, amino acids, flavonoids or lipids (metabolomics) in apple bud samples over a 4-week period prior to flower initiation as identified by bud microscopy. Moreover, functional genomics then describes gene, protein and metabolite functions and interactions. Specifically, large scale omics-profiling was performed using electro-spray ionization mass spectrometry (ESI-MS) coupled with ultra-high performance liquid chromatography (UHPLC) to reveal proteomic and metabolomic differences between vegetative and floral apple buds. Proteomic and metabolic profiling of the samples was carried out at the Core Facility Centre, Unit of Mass Spectrometry, of the University of Hohenheim. Methanol bud extracts for metabolomics were analyzed in two ionization modes, positive and negative. The purpose of double MS-measurement was to maximize the compound detection efficiency. Since plant metabolites differ in their protonation-deprotonation affinities during the ionization, some compounds can be detected only in either the positive or the negative mode. RNA sequencing was conducted with c.ATG at the University of Tübingen on llumina Novaseq. Raw data storage and in-depth bioinformatic analysis was done with the Quantitative Biology Center (QBiC) at the University of Tübingen. Detailed information on all the omics-methodologies are provided in annex 1 to this report.

Outputs

1. Histological analysis of apple buds to reveal flower bud initiation

Identifying the time point of flower bud initiation was a significant prerequisite for a successful multi-omics approach. Consequently, histological bud sectioning was employed to identify the onset and duration of bud initiation in both apple cultivars, 'Fuji' and 'Gala' and to select a four-week period prior to flower bud initiation for the omics-analysis. Furthermore, the effect of crop load and heat accumulation on bud development was also described.

In the 2015 growing season, bud initiation in 'Gala' buds commenced irrespective of treatment at 99 DAFB and commenced until the last sampling date at 127 DAFB. At each sampling time, OFF-trees exhibited a much greater percentage of bud initiation than ON-trees, resulting in average initiation percentages of 87% and 33% for OFF- and ON-trees, respectively. In contrast, bud initiation in 'Fuji' OFF-trees started at 77 DAFB, 22 days earlier than in 'Gala', but in ON-trees only at 120 DAFB, yielding a mean percentage of initiation of 83% for OFF-trees and 17% for ON-trees.

In 2016, the first initiated 'Gala' buds were observed at 126 DAFB, again irrespective of treatment, and nearly one month later than what was found in 2015. Nevertheless, similar to 2015 results, 'Gala' OFF-trees had a 2-fold greater percentage of bud initiation (66%) than ON-trees. Buds from 'Fuji' OFF-trees were first initiated at 84 DAFB, which was close to the time in 2015, whereas buds from 'Fuji' ON-trees were not initiated until the last sampling date at 127 DAFB.

The observed data were used for a logistic regression model, describing the probability of bud initiation in relation to the heat sum with a base temperature of 4°C accumulated from full bloom and expressed as growing degree hours (GDH). The 'Gala' model could only be fitted to the data from 2015 due to sampling in 2016 not covering the bud initiation period. In 2015, the onset of bud initiation, defined as 20% of the maximum initiation rate, occurred in 'Gala' at 21500 GDH (76 DAFB) for OFF-trees and at 28122 GDH (96 DAFB) for ON-trees (Figure 9a). The maximum rate of bud initiation was the same for both treatments (Figure 9b) 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). In contrast, since only one initiated bud was found for 'Fuji' ON-trees, modelling this treatment was not possible. Thus, the onset of bud initiation for 'Fuji' OFF-trees was 15274 GDH (57 DAFB) in 2015 and 19955 GDH (72 DAFB) in 2016. The length of the active initiation period was 53 days in 'Fuji' OFF-trees in 2015 and 2016, respectively (Figure 10a). Again, there was 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) (Figure 10b). 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 and 53 days in 'Fuji' OFF-trees in 2015 and 2016, respectively.

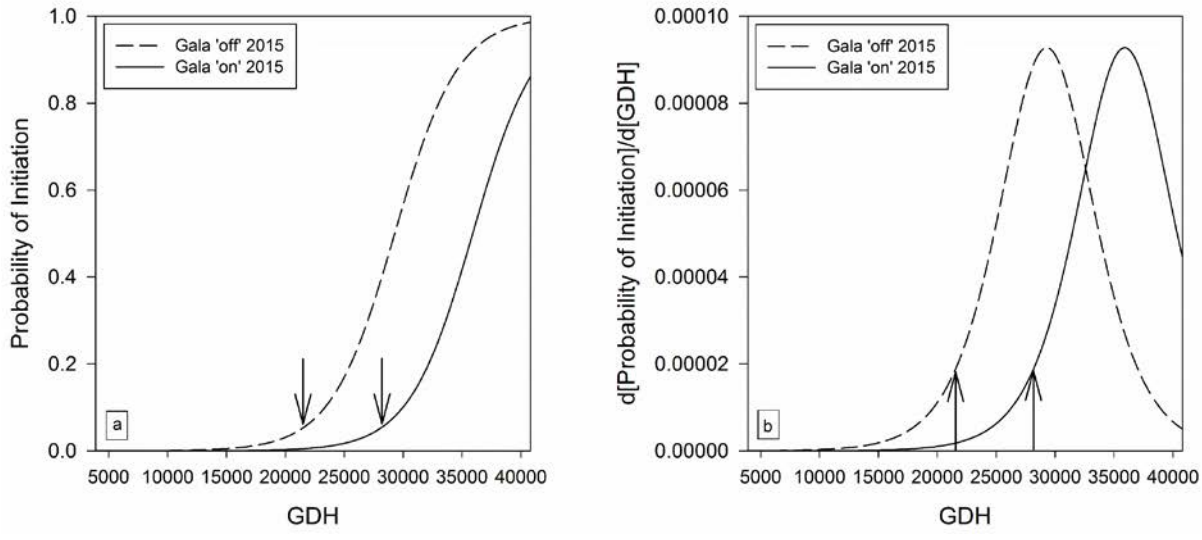


Figure 9. Modelled predictions of the probabilities of bud initiation (a) and the initiation rate (b) in 'Gala' ON- and OFF-trees in 2015. Arrows indicate the onset of bud initiation defined as 20% of the maximum initiation rate: 21500 GDH (76 DAFB) for 'Gala' OFF-trees in 2015; 28122 GDH (96 DAFB) for 'Gala' ON-trees in 2015.

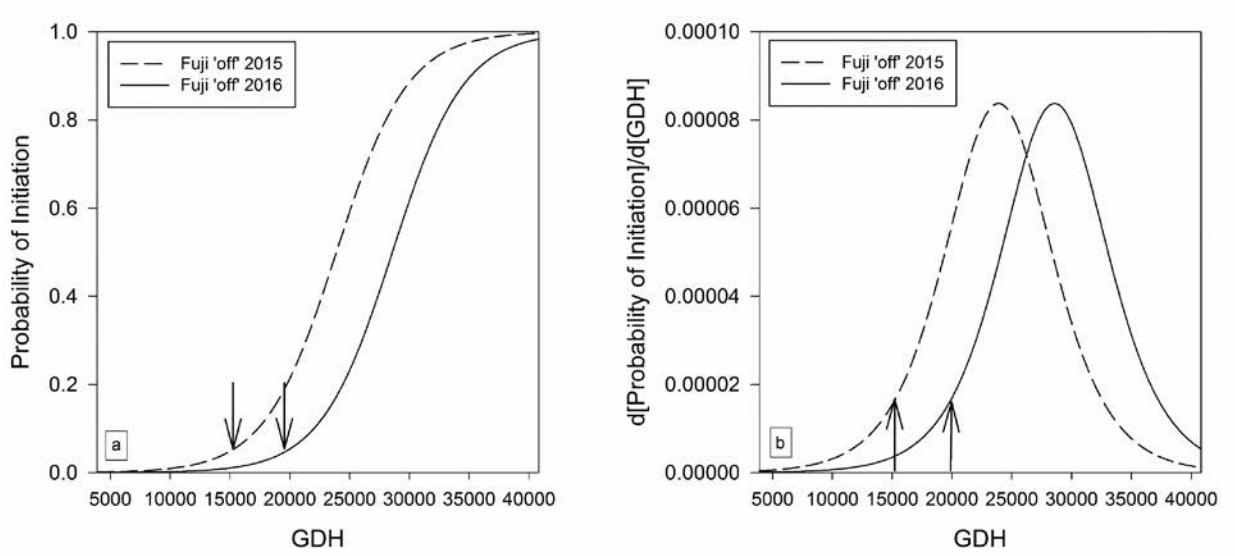


Figure 10. Modelled predictions of the probabilities of bud initiation (a) and the initiation rate (b) in 'Fuji' OFF-trees in 2015 and 2016. Arrows indicate the onset of bud initiation defined as 20% of the maximum initiation rate: 15274 GDH (57 DAFB) for 'Fuji' OFF-trees in 2015; 19955 GDH (72 DAFB) for 'Fuji' OFF-trees in 2016.

Besides the specific identification of the onset of bud initiation, the results of the histological study were summarized in a proposed concept, identifying the main factors influencing the onset of flower bud initiation in apple as shown in Figure 11. In general, the genetic make-up of a given cultivar is the first-level determinant for the onset of flower bud initiation. High crop load (second level determinant) delayed considerably the onset of bud initiation, a response that may occur irrespective of cultivar. It remains unclear whether this crop load driven response in the onset of flower bud initiation is temperature-dependent. Finally, the yearly differences in the onset of bud initiation could be 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.

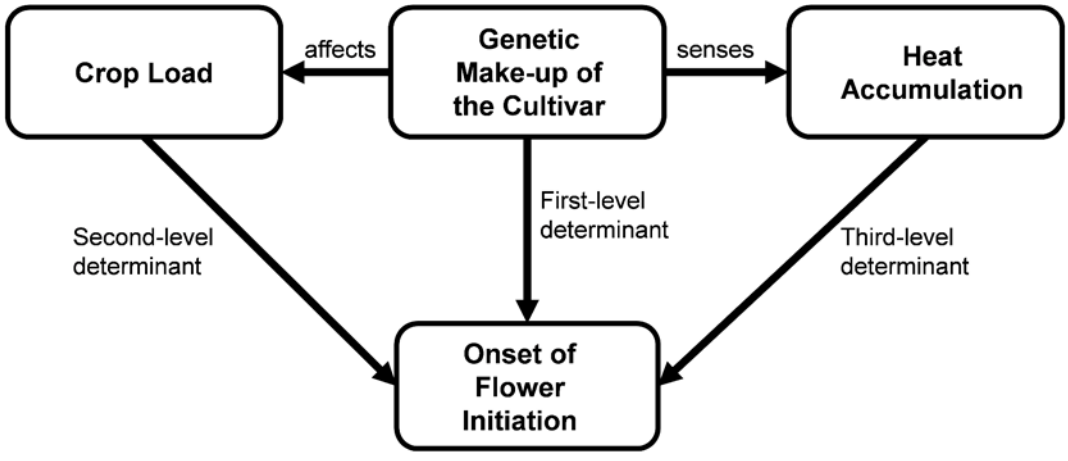


Figure 11. Proposed factors affecting the onset and duration of flower initiation in apple: The genetic make-up of the cultivar, crop load and heat accumulation.

2. Next Generation RNA sequencing and gene expression analysis

Next generation RNA sequencing of 'Fuji' and 'Gala' spur buds revealed 40916 genes (out of 46558 total annotated genes) of which the reads were successfully mapped to the double haploid 'Golden Delicious' genome. After statistical data processing, 6967 genes in 'Fuji' and 3426 genes in 'Gala' showed significant differences in their expression levels between ON- and OFF-trees in any of the four selected sampling weeks, respectively. These genes were used for further computational tests. The number of genes differentially expressed between the treatments was not evenly distributed over the sampling weeks. The majority of genes was up-regulated at 63 DAFB in 'Fuji' and 83 DAFB in 'Gala', which was at similar times for 'Fuji' and 'Gala' OFF-trees when compared to the onset of flower bud initiation that was detected by microscopy.

Comprehensive determination of physiological activity in apple spur buds was achieved by annotating the differentially expressed genes with KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and GO (Gene Ontology) terms. Gene enrichment analysis revealed that spur buds from OFF-trees exhibited more active growth and therefore could reach the stage of flower initiation earlier compared with ON-trees. In the buds from OFF-trees, genes were detected that were related to carbon fixation during cellular photosynthetic activity and fatty acid biosynthesis, where enzymes from pyruvate and biotin metabolism pathways play an important role (Rawsthorne 2002). Fatty acids are then metabolized into different components, including glycerophospholipids, which are used to build cell membranes (Harwood 2005). Moreover, several other physiological pathways were detected, which play a role in amino acid metabolism (phenylalanine, glycine, serine, threonine, tryptophan, arginine, proline, etc.), metabolism of purine and pyrimidine that serve as DNA and RNA constituents, sugar metabolism (pentose and glucuronate interconversions, amino sugar, nucleotide sugar, galactose, starch and sucrose metabolism) and flavonoid biosynthesis. In contrast, enriched physiological pathways in buds from ON-trees differed markedly from those described in OFF-trees. For example, diterpenoid and carotenoid biosynthesis lead to the formation of gibberellins (GAs) and abscisic acid (ABA), respectively. Another sign of plant hormone involvement in the metabolism of ON spur buds was demonstrated by the presence of up-regulated genes controlling signal transduction of at least eight known phytohormone groups: auxins (AUX), cytokinins (CKs), salicylic acid (SA), jasmonic acid (JA), brassinosteroids (BS), ethylene, ABA and GAs. Enrichment analysis of genes up-regulated also revealed some DNA transcription regulators, sequence-specific DNA binding factors and genes coding zinc finger proteins.

From the diversity of sugar metabolic pathways detected in 'Fuji' OFF, the only pathway of the same category identified in 'Fuji' ON was starch and sucrose metabolism. In spur buds of ON- and OFF-trees protecting compounds against pathogens were found as, for example, stilbenoid, diarylheptanoid, gingerol, monobactam and isoquinoline. In addition, biosynthesis of other alkaloids in 'Fuji' OFF and plant pathogen interaction pathway activity in 'Fuji' ON were detected.

In the non-biennial apple cultivar 'Gala' there were less pathways that differed between the spur buds from ON- and OFF-trees. Carbon metabolism, DNA replication and protein synthesis were still attributable to spur buds from OFF-trees, whereas in spur bus from 'Gala' ON-trees, in contrast to 'Fuji' ON, metabolism of membrane lipids (glycerolipids and glycerophospholipids) and phosphatidylinositol signaling system (membrane components with signaling function) were found. Interesting gene activity information was obtained for 'Gala' ON at 83 DAFB, where 12 transcription factors clustered in 2 enriched pathways were found and later, at 89 DAFB, where 4 homeodomain-like genes, which are also classified as transcriptional regulators, were identified.

Following the generally accepted notion that transcription factors and phytohormone regulators are involved in the flower induction mechanisms in apple, the differentially expressed genes were specifically searched for those that, according to their annotation, belong to these two groups. Among these genes, 30 transcription factors and 13 genes regulating plant hormone signal transduction were up-regulated in 'Fuji' OFF, while 56 and 52, respectively, in 'Fuji' ON. In contrast, fewer genes of those groups were found to be up-regulated in 'Gala' with 8 transcription factors and 15 phytohormone-regulating genes in 'Gala' OFF, while 26 and 24, respectively, in 'Gala' ON.

3. Proteomic profiling of apple buds

A total of 7,428 protein species were identified. The average count of identified proteomic compounds per sample prior to all filtering steps was 41,699 for peptides, 17,115 for unique peptides and 3,970 for protein species. After several quality filtering steps, 4,020 protein species, 8.9% of the total apple proteome, remained for detailed bioinformatics analysis.

The Student's T-Tests performed for each sampling date resulted in 159 protein species that were significantly different in abundance between spur bud samples from 'Fuji' ON- and OFF-trees during at least one sampling date. Ninety-three were higher abundant in buds from ON-trees and 66 were higher abundant in buds from OFF-trees.

Cluster analysis presents four clusters of protein species, showing distinct abundance profiles over time. Cluster 132, containing 43 protein species, was more abundant in buds from OFF- than those from ON-trees throughout the entire experimental period with a mean log2FC of -1.4 and a pronounced treatment separation shortly after the onset of bud initiation. Cluster 155 contains 24 protein species with an average log2FC of -0.31; however, there was no difference in abundance during the period of bud initiation. Cluster 46, containing 47 protein species with an average log2FC of 1.4, was more abundant in buds from ON- than those from OFF-trees with a significant difference at 75 DAFB. 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. 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.

The Student's T-Tests performed at each sampling date resulted in 53 protein species that were significantly different in abundance between spur bud samples from 'Gala' ON- and OFF-trees during at least one sampling date. Fourteen were higher abundant in buds from ON-trees and 39 were higher abundant in buds from OFF-trees.

Cluster analysis presents four clusters of protein species, showing distinct abundance profiles over time. Cluster 22 contains 23 proteins and was more abundant at all sampling points in buds from 'Gala' OFF- than those from ON-trees with an average log2FC of -1.66. The abundance stayed relatively constant in OFF-trees but showed a marked decrease at 83 DAFB and 104 DAFB in buds from ON-trees. 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. The abundance decreases markedly in ON-trees at 83 DAFB. 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. The separation between ON- and OFF-buds was occurring from the first sampling date prior to the calculated onset of bud initiation at 76 DAFB. 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.

4. Metabolic profiling of apple buds and apple diffusates

MS-signal computation of metabolic data resulted in 1491 mass-signals (features) in the positive and 796 features in the negative ionization mode. A total of 1140 features with sufficient signal quality and fragmentation spectra could be further characterized. Based on the alignment of fragmentation spectra (MS2-spectra) with existing reference spectra in mzCloud chemical database and precise molecular weight search in ChemSpider, Plant Metabolic Network (PMN) and PubChem, 159 features were linked to potential compounds found in at least one of those databases (pre-identification). After detailed spectra analysis in order to avoid false identifications, the number of pre-identified compounds was reduced to 111. They were characterized with robust MS-signals, sufficient fragmentation spectra and measurable peak areas that were used for further statistical tests.

Statistical data analysis of the pre-identified 111 features resulted in 22 compounds, which differed significantly in abundances between spur buds from ON- and OFF-trees in at least one out of four weeks ('Fuji' 48-68 DAFB and 'Gala' 68-89 DAFB) prior to flower initiation that occurred in 'Fuji' at 75 DAFB and in 'Gala' at 97 DAFB.

Full-scan metabolic dataset provided general information about compound classes that could be detected in apple bud tissue and included amino acids and dipeptides, plant hormone-like acting substances, polyphenols and their glucosides, vitamins, triterpenoids, fatty acids and others, which are not yet included in the chemical databases. The compound identification process is considered to be completed when the structure of a candidate substance is confirmed with the corresponding reference compound. From 22 metabolites, five reference compounds (prolylleucine, thiamine, chlorogenic acid, arginine, trans-3-indoleacrylic acid) were available for purchasing in order to determine if their alignment with the chemical databases was correct. The fragmentation spectra of the reference compounds confirmed the structures of 3 (chlorogenic acid, thiamine and arginine) out of those 5 pre-identified metabolites. Although the MS2-spectrum of trans-3-indoleacrylic acid matched with the one of the standard, the retention times of both did not coincide and therefore the structure of the compound was only partially confirmed.

The fragmentation spectra of polyphenolic compounds such as phloridzin indicated the presence of additional sugar moieties in the molecules (C5-sugar and C6-sugar); however, besides the molecular weights, their structure or their linkages to the aglycones could not be recognized by any of the applied analytical methods.

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 their involvement in other metabolic pathways. Based on the transcriptomic and proteomic data, several metabolic processes assumed to have promoting effects on bud development and meristem differentiation in apple were enriched in spur buds collected from OFF-trees. Specifically, metabolic profiling of 'Fuji' and 'Gala' spur buds showed that amino acids phenylalanine, tyrosine and tryptophan, immediate precursors of the phenylpropanoid biosynthesis pathway that includes biosynthesis of flavonoids and chlorogenic acid in particular, had higher abundance in OFF-trees. Moreover, the average abundance of clorogenic acid was 6 times higher in 'Gala' OFF spur buds in comparison to 'Fuji' OFF spur bud. In previous studies, chlorogenic acid was proven to inhibit IAA-oxidase and therefore to protect IAA 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 this compound might influence bud meristem growth through the interactions with auxins or even act independently having a partial hormonal activity.

Thiamine is frequently used in plant tissue culture as a nutrient that is necessary for 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 this multi-omics study, thiamine biosynthesis pathway could be detected at all data levels, being observed in RNA-Seq dataset, proteomic and metabolic profiles of 'Fuji' and 'Gala' and showing higher abundance in spur buds from OFF-trees. It is yet impossible to conclude whether thiamine contributes to flower initiation by a direct effect on the bud meristem formation or serves as a nutrient for the maintenance of cell function of developing apical meristem tissue.

Tryptophan plays an essential role in plant hormone biosynthesis pathway and serves as a precursor for auxin biosynthesis (Gordon and Paleg 1961). Metabolic profile of 'Fuji' and 'Gala' indicated a presence of a compound that had a fragmentation pattern very close to trans-3-indoleacrylic acid. While the fragmentation of the compound showed many similarities to the one from the reference compound, the retention time of both did not coincide. The potential candidate appeared on the chromatogram much earlier than expected at 3.8 min instead of 18.6 min. We propose that the compound found in apple spur buds had some polar conjugate that made the retention time shift possible. The best candidate that matches the observed parameters is methyl-indole-3-acetic acid (MeIAA).. The results suggest that the average signal intensity of auxin in 'Fuji' ON spur buds was 10 times higher than that in 'Gala' ON buds. Comparison of auxin abundance between spur buds from ON- and OFF-trees within the cultivars showed that in both, 'Gala' and 'Fuji', the compound had higher abundance in spur buds from ON-trees; a result that might have some implication in the underlying mechanism of biennial bearing.

The fact that some compounds were significantly higher abundant in spur buds from ON-trees, led to the assumption that those compounds could move into the buds from the growing fruit, thereby suppressing bud growth and development. In order to test this hypothesis, we compared metabolic profiles obtained from apple spur buds and from apple diffusates. Metabolic profile of 'Kanzi' diffusates resulted in 99 robust MS-signals (features or potential compounds). A total of 65 features were found in both, spur buds and apple diffusates. Following the hypothesis, only those features were selected, which had higher signal intensities in spur buds from ON-trees in comparison to OFF-trees. Cultivar comparison indicated that the auxin-like compound (the compound similar to MeIAA) was the one that differed the most in its signal intensity between the biennial cultivar 'Fuji' and the non-biennial cultivar 'Gala'. Again, this result might explain to some extent the biennial cropping behavior of 'Fuji'.

Metabolic profiling demonstrated that for understanding the complex mechanism of flower bud formation in apple, phytohormone analysis is essential. However, full-scan metabolomics did not cover the full range of known plant hormones because their concentration in the plant tissue is generally very low in relation to other metabolites, thus their MS-signals could not be distinguished from the high background signal noise. To overcome this analytical constraint and to obtain informative data on hormonal activity in relation to flower bud induction in apple, a cooperation with the Leibniz Institute of Plant Genetics and Crop Plant Research in Germany was established. The library of standards included gibberellins, salicylic acid, 21 cytokinin derivatives, 13 derivatives of auxin and 5 of abscisic acid. All the plant hormones were analyzed using MS-method (Multiple Reaction Monitoring). As a result,

The data suggest that young fruit can indeed influence the development of adjacent spur bud by export of various phytohormones. For example, the auxin derivative methyl-indole-3-acetic acid (MeIAA) was detected in apple diffusates and had 1.7-fold higher concentration in 'Fuji' spur buds from ON- compared to those from OFF-trees. Moreover, the concentration of all detected cytokinins was 3 to 9 times higher in spur buds from 'Fuji' OFF than in

spur buds from 'Fuji' ON-trees. ABA concentration was slightly less in spur buds from 'Fuji' OFF-trees than those from 'Fuji' ON-tree, thus the ABA-level in developing buds is unlikely dependent on the presence of fruit.

Based on these results we propose that a cytokinin-auxin balance determines the fate of the developing bud meristems. There is evidence that cytokinin-auxin ratio determines organogenesis of callus cultivated in vitro (Abo El-Nil *et al.* 1976). In apple spur buds, it might have similar function. The cytokinin-auxin ratio in spur buds collected from 'Fuji' OFF-trees was 150:1, whereas in spur buds collected from 'Fuji' ON-trees it was 30:1. Further studies are needed to determine a threshold of this ratio for each cultivar, below which the bud flower meristems cannot be induced.

Outcomes

1. **Histological bud sectioning determined the onset of flower bud initiation in days after full bloom (DAFB) for 'Gala' and 'Fuji' under the growing conditions in Southern Germany. This facilitated the selection of cultivar specific sampling time windows for Next Generation RNA Sequencing, proteomic and metabolic profiling in order to reveal molecular signals, which promote or inhibit flower bud development.**

Flower bud initiation is influenced by crop load, with ON-trees inducing fewer floral buds than OFF-trees within a growing season. The time of transition from vegetative to floral bud meristems was dependent on cultivar and crop load. The first signs of flower bud initiation for the biennial bearing cultivar 'Fuji' were detected on OFF-trees at 70 DAFB, whereas spur buds from 'Fuji' ON-trees did not develop flower meristems. In the regular bearing cultivar 'Gala' the first spur buds with flower meristems were found at 99 DAFB in 2015 and at 126 DAFB in 2016 for both crop load treatments, respectively. Consequently, buds from 'Fuji' OFF-trees committed to flowering at least one month earlier than those from 'Gala' OFF-trees.

2. **Logistic regression modelling of histological data predicted the onset of flower bud initiation in 'Fuji' and 'Gala' and strong relationships were found between flower initiation and both crop load and growing degree hour temperature accumulation, respectively.**

A logistic regression model determined the beginning of bud initiation, defined as 20% of the maximum initiation rate, as follows: 75 DAFB for 'Gala' OFF in 2015; 96 DAFB for 'Gala' ON' in 2015; 57 DAFB for 'Fuji' OFF in 2015; 76 DAFB for 'Fuji' OFF in 2016. 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 occurred 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 day-length. 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.

3. **Using Next Generation RNA sequencing, a complete transcriptome of spur buds from 'Fuji' and 'Gala' with contrasting crop loads (ON and OFF) was obtained. Gene expression analysis indicated that the buds from OFF-trees developed actively and achieved the stage of flower initiation, whereas the growth of spur buds from ON-trees was strongly suppressed. Based on the gene expression pattern, those were selected that could directly or indirectly influence the flower induction processes in apple.**

Next generation RNA sequencing of 'Fuji' and 'Gala' spur buds revealed 40916 expressed genes (out of 46558 total coding genes). A total of 3281 and 2409 genes were up-regulated in spur buds from 'Fuji' OFF and 'Gala' OFF, respectively. Since the expression profiles were obtained under conducive conditions for flower development (without excessive crop load), some of those will be further explored as candidate genes for flower bud induction. Consequently, the apple transcriptome may also be used in future applications aimed at further understanding the mechanisms of flower induction and breeding of regular bearing cultivars.

4. **Proteomic profiling resulted in significant differences of protein species abundance in buds from different cultivars and contrasting crop loads (ON and OFF). Distinct expression patterns of the protein abundance clusters coincided with the onset of bud initiation.**

The proteomics study presents the first large-scale, label-free proteomic profiling of floral and vegetative apple buds during the period of floral bud formation. Sixteen percent (7194 protein species) of the total apple proteome were identified in apple spur buds. After stringent filtering, 212 protein species were identified that were involved in the development of floral and vegetative buds and might be critical for the development of suitable biomarkers for biennial bearing in apple.

5. **Metabolic profiling of apple buds has not yet been described in the scientific literature. The results suggest that the phytohormone auxin, which is synthesized in the young fruit, moves to the adjacent spur buds and suppresses flower bud development. Moreover, the studied apple cultivars differed in fruit auxin biosynthesis, which explains to some extent the cultivar dependent degree of biennial bearing.**

Metabolic profiling of apple spur buds detected a wide range of compounds, which referred to several metabolic processes and pathways. Extensive data analyses showed that phytohormones play essential role in promoting and suppressing of flower bud induction. The results indicated that cytokinin-auxin ratio in spur buds may determine their organogenesis and development of flower bud meristem.

Monitoring and evaluation

The project has progressed well towards its expected outcomes through the delivery of a range of field experiments and laboratory analysis. The short-term outcome of the project was to ensure an improved knowledge by researchers and industry on key plan processes that regulate flowering in apple and impact on biennial bearing. Samples collected from the field experiments conducted in Germany were subjected to a series of laboratory analyses to identify (1) the onset of floral bud initiation, (2) candidate genes which can be used as molecular markers for future breeding activities, (3) specific compounds that modulate flowering behaviour and (4) inhibitory/promoting signals for flowering. These project outputs have been achieved, although we will continue to work on the enormous data set to extract further detailed information at all levels.

The achievement of these outputs was dependent on the adoption and utilisation of modern analytical technologies that have become readily available throughout the project, such as RNA sequencing, proteomics and metabolic profiling. Mass spectrometry (MS) is an analytical technique that has become widely available to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds. The project team have also been utilising this emerging technique in an attempt to identify the metabolite(s) and protein(s) associated with the biennial bearing habit of apple cultivars.

The activities and outputs of the project contributed to the achievement of the medium and long-term outcomes (as specified in the Program Logic). New non-biennial bearing apple varieties are a long-term solution given breeding timeframes. To help growers in the medium term, the project has identified specific compounds that, upon application to a biennial cultivar in an ON or OFF year prior to seasonal flower induction, may reduce or increase flowering gene activity, and thus keep the tree relatively balanced in its annual fruit load. Based on a better understanding of the processes involved in biennial bearing it might also be possible to identify in the medium term more effective crop management practices that reduce biennial bearing in different susceptible apple cultivars. However, more research and engagement with industry will be required to translate these outputs into the development of new varieties and inhibitory/promoting substances that would eventually provide the commercial apple production sector with an excellent horticultural tool to modulate crop levels.

This project is of great relevance to apple growers in Australia and around the world. It is estimated that about 30% of commercial apple cultivars are susceptible to some level of biennial bearing, causing an annual financial loss to growers of about 30-50 Million Euro in Germany. Similar value losses are likely occurring in Australia. Considering that the value of apple production is estimated over $500 million, its estimated that increasing orchard efficiency will improve business profitability by 5% to 10%, which translates to a total improvement of around $25 to 50 million AUD.

The benefits of this project apply to both the science community and apple industry members. Due to the highly technical nature of this project, communication and training has focussed on the communication of scientific results to the research community. This has included the publication of several technical articles and of scientific manuscripts in peer reviewed journals (see section on refereed scientific publications). Industry awareness of this project has occurred by using the key communication tools available to the apple and pear industry which include articles within the Australian Fruitgrower magazine (distribution of 950) and highlights within the e-newsletter ‘Industry Juice’ which has a distribution of 1200. Specifically, this included presentations on research outcomes at the APAL speed updating sessions in 2015 and 2018, participation in a video developed by RMCG and APAL to introduce the PIPS2 projects and two articles in the Australian Fruitgrower Magazine on ‘Finding the triggers of biennial bearing in apples”

A notable feature of the team members is that they are involved in research that stretches across the entire continuum from fundamental and underpinning science through to applied research and technology transfer to growers. It facilitates the application of research findings (regarding plant mechanisms) and services (crop management tools) to alleviate flowering constraints, leading to enhanced orchard productivity and management (long-term outcome). The amalgamation of researchers in Australia and Germany also allows the project to utilise the strengths of each team. The German team have greater access to skills and experience within the academic realm while the Australian team have greater contact and liaison with industry allowing for grower input and feedback into the project. The collaboration between the research teams in Germany and Australia has worked well allowing realisation of the key strengths between the two teams. The German and Australian components

have sufficient scope to operate independently but effective communication between the two has fostered great collaboration. Open and regular communication has been facilitated by email/ skype correspondence, travel between the research sites, and attendance at international events.

The project provided capacity-building opportunities for students and professionals in both Germany and Australia. This included two PhD students in Germany who were involved in the outlined research work packages by taking responsibility for the planning and execution of the various research components. It is expected that both doctoral students will complete the requirements for a PhD degree at the end of 2020.

The project was efficiently coordinated with significant effort made to provide 'value for money' from the funds invested into this research. This has included leveraging of investment from other sources, identification of efficiency gains and sound project management. Research outputs were effectively communicated to the scientific community and industry. Discussion with industry has confirmed the importance of this work: "We lose 20 -30% of our income every year due to biennial bearing. The ability to even out biennial bearing would be extremely valuable and assist with industry wide planning" Kevin Sanders (Orchardist). This project has provided a rigorous scientific body of work, which facilitates the development of practical solutions for the apple industry.

Recommendations

Apple spur and terminal buds are flower formation organs, which are necessary for high bloom density and thus for high commercial yields. In temperate climates, flowering in apple occurs approximately 9 months after the vegetative bud has been induced to a develop into a floral bud in the previous year. However, bud development coincides with active fruit growth, making both developmental processes competing for available tree resources. In fruit trees, the phytohormone auxin is known to be synthesized in apical shoot meristem and its basipolar transport prevents the lateral buds from outgrowth and branching. Auxin is also synthesized in seeds of young apple fruit and the polar auxin transport away from the fruit leads to the inhibition of the subtending spur bud by suppressing flower bud development. However, the total concentration of auxin transport out of fruit and thus the effectiveness of inhibiting the transition from a vegetative to a floral bud meristem seems to be cultivar specific. These differences might be associated with one of the underlying mechanisms that make an apple cultivar biennial such as 'Fuji' or non-biennial such as 'Gala'. Moreover, another group of phytohormones, cytokinins, which are predominantly synthesized in roots and transported to growing plant organs, have a promoting effect on flower bud induction. Flower and fruitlet thinning removes auxin-rich plant organs and thereby facilitates flower bud development to attain a relatively high and consistent crop in the following year. In addition, considering that a high cytokinin-auxin ratio is essential for bud organogenesis, spray applications of synthetic cytokinins should also promote flower bud induction. The current study focused on the physiological, metabolic and molecular basis of biennial bearing in apple, which will in the medium- to long-term result in specific recommendations for the apple industry in Australia and worldwide. It is particularly expected that information on genes that trigger biennial bearing may lead to breeding of non-biennial bearing apple cultivars and that the identification of compounds that affect floral integrator gene activity and thus the transition to flowering in apple trees may lead to the commercial applications for regulating flowering.

Refereed scientific publications

Fruit grower journals

1. Wünsche J.-N., Kofler J., Milyaev A., Flachowsky H., Stefanelli D., 2017. Finding the triggers of biennial bearing in apples. *Australian Fruit Grower Magazine*, December 2016/ January 2017, pp. 30-31, and *the APAL newsletter* https://apal.org.au/finding-the-triggers-of-biennial-bearing-in-apples/.
2. Wünsche J.-N., Kofler J., Milyaev A., Flachowsky H., Stefanelli D., 2019. Finding the triggers of biennial bearing in apples. *Australian Fruit Grower Magazine*, Summer 2019, pp. 44-45.

Grower seminars

1. Wünsche J.-N. Physiological, metabolic and molecular basis of biennial bearing in apple. APAL speed updating sessions in 2015, linked by video conference.
2. Wünsche J.-N. Consistency instead of irregularity: Biennial bearing in apple. APAL speed updating sessions in June 2018, Brisbane, Australia.
3. Wünsche J.-N. Key limiting factors to productivity and fruit quality of apple. APAL conference in June 2018, Brisbane, Australia.

Video

1. Wünsche J.-N. Participation in a video developed by RMCG and APAL to introduce the PIPS2 projects on “Physiological, metabolic and molecular basis of biennial bearing in apple”. June 2018, Brisbane, Australia.

Scientific abstracts

1. Kofler J., Milyaev A., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2016. The physiological, metabolic and molecular basis for biennial bearing in apple. First European Conference of Postgraduate Horticultural Scientists, Palermo, Italy.
2. Milyaev A., Kofler J., Stefanelli, D., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2017. Apple bud histology: A tool to study floral bud development in relation to biennial bearing. Symp. on Flowering, Fruit Set and Alternate Bearing, Palermo, Italy.
3. Kofler J., Milyaev A., Pfannstiel J., Stefanelli, D., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2017. Biennial bearing in apple: Proteomic profiling of developing buds. Symp. on Flowering, Fruit Set and Alternate Bearing, Palermo, Italy.
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1. Milyaev A., Kofler J., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2017. Apple bud histology: A tool to study floral bud development in relation to biennial bearing. *DGG-Proceedings*, Vol. 7, No. 7, pp. 1-5.
2. Kofler J., Milyaev A., Pfannstiel J., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2017. Biennial bearing in apple: Proteomic profiling of developing buds. *DGG-Proceedings*, Vol. 7, No. 8, pp. 1-5.
3. Stefanelli D., Plozza T., Flachowsky H., Wünsche J.-N., 2018. Young apple tree responses to crop load. *Acta Horticulturae* 1229, pp. 221-228.
4. Milyaev A., Kofler J., Pfannstiel J., Stefanelli, D., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2018. Histological and proteomic approaches to study floral bud induction in relation to biennial bearing in apple. *Acta Horticulturae* 1229, pp. 277-284.
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1. Kofler, J., Milyaev, A., Capezzone, F., Stojnić, S., Mićić, N., Flachowsky, H., Hanke, M.-V., Wünsche, J.-N., 2019. High crop load and low temperature delay the onset of bud initiation in apple. *Scientific Reports* 9, 1-11.
2. Milyaev, A., Kofler, J., Klaiber, I., Czemmel, S., Pfannstiel, J., Flachowsky, H., Stefanelli, D., Wünsche, J.-N., 2020. Flower induction in apple: Multi-omics approach. Submission is scheduled by the end of June 2021.
3. Kofler, J., Milyaev, A., Wuertz, B., Pfannstiel, J., Flachowsky, H., Wünsche, J.-N., 2020. Proteomic differences in apple spur buds of ON and OFF trees during floral induction. In submission.

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Intellectual property, commercialization and confidentiality

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Appendices

Methodologies

1. **ANNEX 01.** Methodologies of histological bud sectioning and microscopy, Next Generation RNA sequencing, proteomics and metabolic profiling.

Popular articles for grower magazine

1. **ANNEX 02.** Wünsche J.-N., Kofler J., Milyaev A., Flachowsky H., Stefanelli D., 2017. Finding the triggers of biennial bearing in apples. *Australian Fruit Grower Magazine*, December 2016/ January 2017, pp. 30-31, and *the APAL newsletter* https://apal.org.au/finding-the-triggers-of-biennial-bearing-in-apples/.
2. **ANNEX 03.** Wünsche J.-N., Kofler J., Milyaev A., Flachowsky H., Stefanelli D., 2019. Finding the triggers of biennial bearing in apples. *Australian Fruit Grower Magazine*, Summer 2019, pp. 44-45.

Abstracts

1. **ANNEX 04.** Kofler J., Milyaev A., Flachowsky H., Hanke M.-V., Wünsche J.-N. 2017. The physiological, metabolic and molecular basis for biennial bearing in apple. First European Conference of Postgraduate Horticultural Scientists, Palermo, Italy.
2. **ANNEX 05.** Kofler J., Milyaev A., Pfannstiel J., Stefanelli, D., Flachowsky H., Hanke M.-V., Wünsche J.-N. 2017. Biennial bearing in apple: Proteomic profiling of developing buds. Symp. on Flowering, Fruit Set and Alternate Bearing, Palermo, Italy.
3. **ANNEX 06.** Milyaev A., Kofler J., Stefanelli, D., Flachowsky H., Hanke M.-V., Wünsche J.-N. 2017. Apple bud histology: A tool to study floral bud development in relation to biennial bearing. Symp. on Flowering, Fruit Set and Alternate Bearing, Palermo, Italy.
4. **ANNEX 07.** Flachowsky H., Weigl, K., Djurić, G., Mićić, N., Garkava-Gustavsson, L., Zborowska, A., Si-Ammour, A., Asquini E., Sotiropoulos, T., Wünsche J.-N., Hanke M.-V. 2017. European study on the time of flower induction in apple. Symp. on Flowering, Fruit Set and Alternate Bearing, Palermo, Italy.
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1. **ANNEX 10.** Milyaev A., Kofler J., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2017. Apple bud histology: A tool to study floral bud development in relation to biennial bearing. *DGG-Proceedings*, Vol. 7, No. 7, pp. 1-5.
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3. **ANNEX 12.** Milyaev A., Kofler J., Pfannstiel J., Stefanelli, D., Flachowsky H., Hanke M.-V., Wünsche J.-N., 2018. Histological and proteomic approaches to study floral bud induction in relation to biennial bearing in apple. *Acta Horticulturae* 1229, pp. 277-284.
4. **ANNEX 13.** Stefanelli D., Plozza T., Flachowsky H., Wünsche J.-N., 2018. Young apple tree responses to crop load. *Acta Horticulturae* 1229, pp. 221-228.
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