Horticulture Innovation Australia

Final Report

Bridging the knowledge-gap to breed high-value, flavonoid-rich apples

Michael Considine
Western Australian Agriculture Authority (WAAA)

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Sydney NSW 2000
Tel: (02) 8295 2300
Fax: (02) 8295 2399

Content

Summary

Apples are consistently among the top three fresh fruits consumed worldwide and the top 20 agricultural crops. Their contribution to local and global dietary health is becoming irrefutable, and increasing evidence points to significant benefits in cardiovascular function from eating apples, even in otherwise healthy consumers. Consumers are increasingly aware of the health qualities of foods, as evident in social media and the growing global value of functional foods. However, fresh fruits and vegetable industries have failed to capture the value of this knowledge and opportunity. Recent changes to the national health claims legislation have lowered the barrier to making an application for higher level health claims for apples and other fresh produce. This project sought to break down other practical and knowledge barriers to determine the capacity and viability of breeding and marketing new flavonoid-rich apples. The primary gaps this project sought to address were:

1. Can we breed a flavonoid-rich apple, if given the tools to identify it?
2. Can we identify a market and particularly a pathway to that market?

Following two desktop reviews that examined the genetic and cultural influences on flavonoid composition and content in apples, the research team established research into the genetic variation of flavonoids in varieties and breeding accessions within the Australian National Apple Breeding Program (ANABP), Manjimup WA. Analysis showed considerable variation in type and amount of flavonoid amongst varieties, considering quercetin, epicatechin, chlorogenic acid, phloridzin and anthocyanins. Some comparisons revealed >100-fold range; for example 0.2-20 mg/100g epicatechin in the whole fruit. Significantly, the range of flavonoid content of ANABP germplasm compared favourably with other naturally flavonoid-rich foods associated with health, including grape seeds, prune juice, green tea and coffee. Considering that each of the ANABP selections had already passed stage 1 selection, the genetic potential within ANABP is likely to be considerably greater.

Pedigree relationships were used to calculate narrow-sense heritability of each class of flavonoid, and estimated breeding values of each genotype. These data strongly support the capacity to selectively breed apples with elite levels of one or more flavonoid. Reduced-representation genotyping was carried out, however preliminary analysis demonstrates the need to carry out genome-wide analysis to establish a genomic estimates of breeding values. These data are the necessary next step towards implementing marker-assisted selection for flavonoid content in apples. Coupled with rapid breeding technology, the research team believe accelerated breeding and selection of new flavonoid-rich apples is achievable within 5-10 years.

Towards understanding the market, the research team carried out three research strategies: a survey of social media; unbiased local consumer panels, and; targeted online surveys designed for conjoint analysis. The social media data reinforced the strong associations of apples to health, fitness, diet and antioxidants. Nevertheless, consumer panels indicated that the principal drivers of purchase were price and appearance. Conjoint analysis seeks to partition primary influences in purchase decisions. Price was again found to be most influential, however the analysis indicated that antioxidants were an important consideration in purchase decisions, and more-so than organic status. Further analysis is required, particularly to establish the degree to which consumers relate colour to a healthy value, as national nutrition campaigns have promoted.

An important strategic consideration for marketing is the use appropriate and considered terminology. In designing market research, it was apparent that the term flavonoid was not widely known or understood. Yet the term antioxidant is largely meaningless and its continued use poses a risk to loss of faith from consumers. This reality has resulted in the USDA withdrawing its food antioxidant database, and several manufacturers removing the term antioxidant from packaging. However, our own analysis confirms that it is widely and positively accepted by local consumers at present. One of the recommendations of the review team is a considered national discussion.

Taken together, this project has made a considerable step-change towards selectively breeding and marketing flavonoid-rich apples, and growing value for industry. We recommend that industry support an application for a

higher-level health claim associated with cardiovascular benefits, and support the necessary next steps to establish and implement marker assisted selection for elite flavonoid levels in Australian apples. The global functional food market exceeds that of apples, yet in many cases products within the category have less functional evidence in dietary health than apples. Hence we see the opportunity to add value is considerable. More broadly, we see that this strategy and opportunity is not limited to apples or flavonoids, and hence we recommend establishing a national training centre incorporating the multiple disciplines and skills required to develop and sustain a more clever health-driven fresh fruit market.

Keywords

Flavonoid, dietary health, cardiovascular disease, health claims, antioxidant, breeding, genetics, market research.

Introduction

Apples and other fresh fruits compete within the snack market and hence against a range of packaged foods. Increasingly, many packaged snacks are fortified with health-promoting compounds or in other ways marketed as 'healthy', providing advantages in nutrient and health labelling. While the evidence of nutritional and other health benefits of fruit intake is conclusive, this has failed to translate to a competitive value for industry. This raised the fundamental question; if apples are known to be healthy and we can identify particular phytochemicals, can we selectively breed and market new 'healthier' varieties to drive increased value for industry? The experience of a number of projects addressing similar questions has identified particular knowledge gaps in addressing this question and the market opportunity. The primary gaps this project sought to address were:

1. Can we breed a flavonoid-rich apple, if given the tools to identify it?
2. Can we identify a market and particularly a pathway to that market?

Breeding potential

Apples contribute a number of health benefits, which have been recently reviewed [1]. Apples are rich in flavonoids and phenolic acids, notably quercetin glycosides (flavonols), (-)epicatechin and (+)catechin (flavan-3-ols) phloridzin, chlorogenic acid and anthocyanins, each of which have demonstrated functions in dietary health [2, 3]. Studies have identified a number of sources of variation in flavonoid content in fruits, including variation between varieties. However, there has not been a comprehensive review of the role of genetic variation or capacity for breeding new varieties with targeted levels of flavonoids. In addition, fruit composition varies with pre- and postharvest conditions, and to date there has been no review of this knowledge in relation to flavonoids in apple. Hence the first aim was to review and publish two papers on the genetic and pre- and postharvest variation in flavonoids in apples.

Developing and marketing new varieties is a critical strategy for generating value in fruit industries. Australia has a competitive history of breeding and exporting market-leading apple varieties. The Australian National Apple Breeding Program (ANABP) has generated the global successes Cripps Pink (Pink Lady™), Cripps Red (Sundowner™) and the recent ANABP 01 (Bravo™), each based on selection for desirable consumer traits (colour, flavour, texture). The next frontier for fruit breeding is generating more nutritious varieties, with elite levels of phytochemicals. However the time and resources required to bring novel varieties to market by traditional plant breeding methods is prohibitive. The germplasm and resources of the ANABP provided opportunities to test the potential to breed and market (below) health-enriched varieties. The second aim was to characterise the variation in flavonoid content between *ca* 100 commercial varieties and breeding lines in the ANABP with known pedigree relationships, and quantify breeding values for designing future crosses. In addition, we carried out genetic fingerprinting to identify genetic markers associated with flavonoid content.

Marketing potential

The global functional food market exceeds US$250 billion, *ca* five times the GVP of apples [4, 5]. Capturing some of this value for fresh products has been challenging, as illustrated by the Vital Vegetables project [6]. Hence the third aim was to carry out market research to identify the relative importance of health (flavonoids) as a factor in decision-making when buying apples. This considered a survey of social media, unbiased buying preferences of consumer panels (without knowledge of the project aims) as well as targeted online surveys taking into account demographics. The consumer panel analysis considered a hypothetical 'new' flavonoid-rich apple, using the recently developed ANABP 01 (Bravo™). The targeted surveys were designed for a conjoint analysis, considering the relative importance of number of attributes (price, flavour, colour etc) in purchase choice. In addition, we sought to establish an online information portal explaining and promoting the health benefits of apples, directly related but not limited to the ANABP.

On commencing this project, the legislative environment for marketing products with health claims was restricted. The Food Standards Australia New Zealand (FSANZ) have since expanded the health claims framework to allow new applications for higher level health claims [7]. With the advances made through this project and associated research, the apple industry are now poised to capture that opportunity through establishing a multidisciplinary strategy to seek a higher level health claim, rapidly breed new flavonoid-rich apples, and build market value for the apple industry. Developing and implementing this strategy will provide a pathway to extend the opportunity to other fresh fruits and vegetables as well as other consumer traits.

Methodology

Aim 1. Review genetic and cultural variation in flavonoid content in apples.

Two desktop studies were designed, aimed at providing information for the public, preventing duplication and shaping strategies for further research. Both reviews were invited by an international publisher to be published in a peer-reviewed and edited book series “Novel Postharvest Treatments of Fresh Produce (A volume in series 'Innovations in Postharvest Technology')”, CRC Press.

The review of genetic variation considered the information available in all pome fruits and extended the context to other flavonoids, anthocyanins and polyphenols in order to guide an understanding of whether the heritability of flavonoids was reasonably simple, governed by a number of structural genes or pathways, or whether the trait was complex, comprised of a number of genetic pathways and regulatory inputs. The review of pre- and postharvest variation considered variation across plant foods, and the primary cultural influences on flavonoid, anthocyanin and polyphenol content in apples, and summarised the important considerations and opportunities to maximise flavonoid levels in a given variety.

Aim 2. Characterise genetic variation in flavonoid content Australian-bred apples.

Apple fruit from 72 breeding accessions (Stage 2) and 19 cultivars were included in the study, all sourced from the Australian National Apple Breeding Program site in Manjimup, Western Australia (32.2°S, 116.1°E). Apple cultivars included Big Time, Cripps Pink, Cripps Red, Firm Gold, Fuji, Gala, Galaxy (red selection of Gala), Golden Delicious, Granny Smith, Hi-Early (selection of Red Delicious), Lady Williams, Naga Fu No. 2 (red selection of Fuji), Purple Wave (crab apple), Red Braeburn (red selection of Braeburn), Sansa, Splendour, Wandadale, Western Dawn, and Western Tang. Apples were harvested at storage maturity in March and April 2015. All fruit were grade 1-equivalent quality. At least 4 fruit per genotype were sampled from a minimum of two trees and from a range of positions on the tree. The apples were transported to the University of Western Australia and upon arrival apples were stored at 4°C for up to 8 weeks. Whole apples were weighed individually to determine an average weight of each variety. 10 g of apple skin only and 10 g of apple flesh only were taken from different surface areas of the 4 apples for each variety from each tree. Flavonoid content was assayed by HPLC, details are provided in a manuscript submitted for publication.

In order to relate variation to pedigree, breeding values were calculated from the flavonoid composition of each variety. The pedigree and phenotypic records for each genotype were recorded, with measurements on several replicate apples from each genotype. A pedigree tree was visualised using Pedimap [8]. All statistical models were fitted using statistical software ASReml-R (v. 3.0) [9], which produces residual maximum likelihood (REML) estimates of the variance parameters and best linear unbiased predictions (BLUP) of the random effects. A relationship matrix was formed based on pedigree information. Both additive (predicted breeding values correlated according to the relationship matrix) and non-additive (uncorrelated) genetic effects were included in the mixed model for each trait (polyphenolic concentration in flesh and peel). Variance components were estimated for additive and non-additive effects, and narrow-sense heritability was calculated for each trait as the ratio of the additive variance component divided by the sum of additive, non-additive and error variance components. In nearly all cases, partitioning the genetic variance into additive and non-additive (uncorrelated) components using the pedigree-based relationship matrix significantly increased the log likelihood of the mixed model.

Diversity Arrays Technology-sequencing (DArTseq) was carried out in order to enable a pathway to identifying important regulatory features of the genome. Freeze-dried leaf samples from 91 trees from the ANABP were submitted to DArT Pty Ltd for DArTseq analysis (a form of genotyping-by-sequencing [10]). A total of 118 samples were analysed including 27 technical replicates. The resulting set of single nucleotide polymorphism (SNP) markers were subjected to quality filtering, removing SNP markers with reproducibility <0.95 and call-rate <0.5. Genetic relationships among the trees were assessed using filtered DArTseq-derived SNP markers. Pairwise Euclidean distances were calculated using NTSYSpc 2.21i (Applied Biostatistics Inc.), which were then subjected to hierarchical cluster analysis using group averages in Primer 6.1.6 software (Primer-E Ltd).

Aim 3. Identify the relative importance of health (flavonoids) in apple purchase decisions.

These studies were undertaken by the Co-operative Enterprise Research Unit (CERU) at UWA. Three studies were designed, each to inform the subsequent one:

i. *Survey of social media sites and blogs related to healthy eating*.

   The aim of this step was to identify how apple consumers think, for example priorities, and attributes that they consider when purchasing or eating apples. A number of relevant Australian-based forums were explored.

ii. *Focus groups with consumers who purchase apples in WA to explore their attitudes towards food, healthy eating and the consumption of fresh fruit*.

   The aim of these focus groups was to examine consumer attitudes towards apples and help identify the main attributes that motivate purchase of apples. It was designed to be unbiased, with volunteers given no prior knowledge of the aims of the study. However with the recent release of ANABP 01, it was decided to include ANABP 01 and Cripps Pink, introducing ANABP 01 as a hypothetical new healthy ('flavonoid-rich') apple.

   Six focus groups were held where consumers from a range of backgrounds discussed healthy eating, fruit purchasing and consumption, apple preferences, flavonoids and the new ANABP 01 apple. These focus groups included a taste testing and comparison between the Cripps Pink and ANABP 01 apples. The participants in the focus groups were recruited by a professional market research data collection agency and drawn from their large consumer panel. They were selected to provide a good representative sample of the general population and included a wide range of ages, family, education, income and lifestyles. The focus groups took place over one and half hours and followed a common discussion guide that commenced with a general discussion over healthy diet and eating, the role of fruit, knowledge of apples, purchase and consumption behaviour in relation to apples, knowledge of flavonoids, and a final taste testing of the Cripps Pink and ANABP 01 apple varieties. The data from the transcripts were analysed using the NVIVO qualitative data analysis software.

iii. *Conjoint experiment via an online consumer panel*.

   The attributes identified in the focus groups were built into an online experiment among apple purchasers where the respondents are asked to rate their preference for a range of combinations of price, taste, health benefit etc. as determined from the preceding stages. This generated a list of options that were preferred by most or some market segments. A pilot experiment surveyed 200 respondents to test and refine the design prior to a surveying 600 additional participants.

Online education of health properties of apples

In addition, we undertook to establish an online information portal, recognising considerable range in consumer knowledge of the health benefits of apples, and the underlying factors contributing and affecting this. We acknowledge that the national industry, via Horticulture Innovation Australia have undertaken to improve consumer knowledge via the website www.aussieapples.com.au. We sought to establish a local (WA) web page, which expanded on information from the Aussie Apples page and in particular information relevant to breeding.

Outputs

Aim 1. Review genetic and cultural variation in flavonoid content in apples.

Two reviews were accepted for publication, see Scientific refereed publications

Interested parties should contact Michael Considine directly for an electronic copy michael.considine@agric.wa.gov.au or michael.considine@uwa.edu.au.

Summary of "Flavonoids in pome fruits: health benefits, genetic control and prospects to breed an even healthier apple".

This chapter summarised flavonoid health benefits, genetics and improvement in pome fruit breeding. The purpose of this review was to foster communication between researchers and practitioners spanning pome genetics and breeding, plant biochemistry, the pharmacology and clinical health, and marketing and commerce, in order to guide crop improvement for more enriched, healthier product to market.

Wide variation in flavonoids in pome fruits among the cultivars/genotypes provides breeders opportunity to manipulate optimal levels of flavonoids in the fruits; increasing the flavonoid content. Successful examples of manipulating flavonoid concentration in many crops have showed that this goal can be achieved by traditional breeding with/without marker assisted selection and genetic modification, though there are pros and cons for each approach.

The ability to breed and select for flavonoid content or composition using traditional breeding approaches is currently restricted by (i) a lack of fast, simple and reliable flavonoid phenotyping tools, and; (ii) a long generation time and juvenile development before fruiting. Identification and quantification of flavonoids is presently limited to lab-based approaches and desktop equipment such as HPLC. While not beyond the technical or financial reach of a breeding program, implementing flavonoid analysis through later validation or quality assurance is currently beyond reach. This may be one of the major practical barriers to the longer term success of creating value from flavonoid-rich fruits.

Genetic modification of the flavonoid biosynthetic pathway offers an effective approach to improve flavonoid content in fruits. However, this is challenging politically and technically. The political challenge is not discussed here, but the technical challenges extend to identifying tractable transformation processes, as well as important regulatory genes. Although the structural flavonoid synthetic pathway is known, it is largely regulated by transcription factors that impose control on rate-limiting steps. The important regulatory control of MYB-type transcription factors on flavonoid synthesis has been demonstrated in other plant and crop species. Identifying the rate-limiting transcription factors or other controls is not a trivial challenge. Genome-wide association studies (GWAS), coupled with pedigree analysis, offer holistic and resolute approaches to identify important regulatory genes.

Molecular marker assisted selection, coupled with rapid-breeding approaches, offers a means to manipulate flavonoid by accelerating traditional breeding and selection, rather than genetic modification. Rapid breeding systems have been developed for pome fruit, enabling fruit quality traits to be identified in 1-2 years cf 4-5 years. The use of GWAS and pedigree analysis to identify candidate genetic markers for fruit flavonoid content that can be implemented in breeding programs is a major priority. The second priority is the development of accurate, portable and cheap flavonoid analyses for use in the field or packing house.

Summary of "Maximising the levels of dietary flavonoids in apples and Rosaceae".

This review sought to summarise the pre- and postharvest variation in flavonoid content and composition in Rosaceae fruits, particularly apple, and identify the key handling conditions and storage environments necessary to maximise flavonoid levels before harvest and to minimise any loss of flavonoids postharvest. There are over 5000 flavonoids in plants and several dozens of relevant flavonoids in apples and other Rosaceae fruits. There was evidence of quantitative and qualitative effects on flavonoid composition due to pre- and postharvest treatment, however this degree of variation was not as great as found between variety or species. Growing conditions (*i.e*. light, trellising, pruning of leaders and nutrition) and optimal ripeness were key variables, as flavonoid content develops and changes during fruit ripening. There was little evidence of decline under a standard postharvest cool chain. We also explored pre- and postharvest treatments which may be used to enhance or preserve flavonoid content. The exposure to UV-B or natural light appears to be important in production of flavanone and anthocyanin biosynthesis. Postharvest treatments such as 1-MCP, AVG and high pressure sodium lights also showed evidence of beneficial effect. However, by and large pre-harvest conditions had greater cultural influence on flavonoid levels than postharvest conditions. As such, when stored using sound postharvest practice, flavonoid levels were maintained for at least three months. Storing fruit for longer periods can be more problematical in terms of quality and flavonoid levels. Nevertheless, it is important to consider whether these effects on flavonoid content are significant in relation to the amount required for bioactive effect on dietary health. This will be borne out by further cross-disciplinary research between horticultural and nutritional/medical researchers.

Aim 2. Characterise genetic variation in flavonoid content Australian-bred apples.

This study involved screening the fruit of 91 genotypes for flavonoid composition and content, as well as pedigree analysis using that data and genetic analysis of each of the genotypes. This was used to explore the range and diversity of flavonoid profiles in apples, both qualitative and quantitative, and estimate the degree of genetic control over these traits. One manuscript from the flavonoid composition data has been submitted for publication in Food Chemistry (Bondonno *et al*., see Scientific refereed publications); key data are summarised below. A manuscript from the genetic and pedigree analysis is in preparation; key data are summarised below. In addition, a summary of these data were presented at the 9th International Workshop on Anthocyanins, Auckland NZ, February 2017 and will also be presented at the N8 Agrifood 2017 International Sustainable Food Production Conference, Durham UK, July 2017 and Fourth International Horticulture Research Conference, East Malling UK, July 2017. An abstract has also been submitted to the Great Southern Great Science Symposium, Albany WA, August 2017.

Flavonoid composition

From Bondonno *et al*. (submitted):

Using high performance liquid chromatography we quantified quercetin glycosides, (-)-epicatechin, chlorogenic acid, phloridzin and anthocyanins in skin and flesh of 19 cultivars and 72 breeding accessions from the Australian National Apple Breeding program. Considerable variation in concentration of phenolic compounds was found between genotypes: quercetin (16.1±5.9, range: 5.8-30.1 mg/100g); chlorogenic acid (11.3±9.9, range: 0.4-56.0 mg/100g); (-)-epicatechin (8.6±5.8, range: 0.2-19.8 mg/100g); phloridzin (1.1±0.6, range: 0.3-4.3 mg/100g) and anthocyanins (1.8±4.4, range: 0-40.8 mg/100g). All phenolics except chlorogenic acid were more concentrated in the skin, with total skin concentration approximately 30-fold greater than in the flesh.

Table A1 in the **Confidential Appendix** was extracted from Bondonno *et al*. (submitted). Even among the top five commercial varieties in Australia, the concentrations of key dietary flavonoids vary enormously; >4-fold in quercetins, 10-fold in epicatechin, 1.5-fold in chlorogenic acid, 5-fold in phloridzin and >20-fold in anthocyanin (based on whole fruit values). In addition, the data from ANABP breeding lines demonstrates considerable genetic variation, as suggested by Shan *et al*. (2017). Although Cripps Pink is equivalent to the best accession on a basis of quercetins, it is at least 0.5-fold below the best breeding accession on the basis of the other classes of flavonoids. This clearly illustrates the opportunity to use a targeted approach to breeding and identifying lines with elite levels of flavonoids across all classes. Furthermore, it is important to note that all of the ANABP lines assessed in this study had passed strict selection based on desired consumer and agronomical traits including size, colour, taste, texture, growth habit and yield. Variation due to environment, seasonality or postharvest need to be considered, however research suggests these effects are less influential on flavonoid content than genetics (Seaton *et al*., 2017; Bondonno, Considine and Hodgson, data not shown). Hence this table understates the genetic potential to breed elite lines from the ANABP.

Flavonoid-based pedigree analysis

By combining known pedigree relationships with the flavonoid levels of each genotype, we were able to determine estimated breeding values, which indicate the potential to breed enhanced lines for each of the flavonoid classes, as well as indicate which lines are ancestrally strong contributors to those breeding values, *i.e*. which lines would be most valuable parents in future crosses for elite flavonoid levels. The data are in preparation for publication, and summarised in the **Confidential Appendix**. The data indicated that epicatechin and phloridzin were highly heritable (narrow-sense heritability >0.6) in both skin and flesh, as was anthocyanin in the skin (not determined in flesh). No single variety had high estimated breeding values for all flavonoids measured, further indicating differing genetic control between flavonoids.

In addition, genetic analysis was undertaken on these genotypes in a step towards identifying molecular markers for fruit flavonoid content. A total of 91 trees were genotyped at 30028 single nucleotide polymorphism (SNP) alleles representing 15014 loci. Quality filtering removed 4121 alleles, leaving 25907 SNP alleles for genetic diversity analyses. A dendrogram illustrating genetic relatedness based on these markers is provided in the **Confidential Appendix**. Data are being further interrogated towards quantifying genomic estimates of breeding value (GEBV) for flavonoid composition and content.

Aim 3. Identify the relative importance of health (flavonoids) in apple purchase decisions.

A series of studies were carried out and a summary of key data and summaries are presented here. Manuscripts are in preparation to be published. Summaries of each are provided below and further data provided in the **Confidential Appendix**.

i. *Survey of social media sites and blogs related to healthy eating.*

The aim of study was to identify key concepts around healthy eating, based on the content searched for by Australia consumers and posted to social media by Australian consumers. An investigation was undertaken into a range of social media channels including Twitter, Instagram and Blogs.

The study identified a number of known and novel trends, for example that consumers are interested in the health benefits of food, as well as its role in supporting fitness or lifestyle, plus the general convenience of foods. By and large, women were more likely to use social media for search terms associated with ‘healthy eating’, ‘healthy food’ or ‘healthy diet’. The level of consumer interest in ‘antioxidants’ was investigated was associated with issues such as ‘gluten free’, ‘clean eating’, ‘healthy’, ‘paleo’ and ‘vegan’. The terms ‘fruit’ and ‘apple’ were tightly associated with related health and fitness terms, as well as freshness, taste and convenience. The term ‘apple’ was also associated with some other foods such as coconut and dark chocolate, as well as location issues like Australia and farmers.

The findings were used as a foundation for the development of the focus groups in the second stage. They suggest a relatively strong interest in healthy eating in order to promote health and fitness, as well as for specific lifestyles (*i.e*. Vegan or Paleo diets). A clear gender divide also emerged with women more likely to focus their attention on healthy eating to assist with dieting, while men were more likely to focus on specific issues such as antioxidants and apples. Overall, apples were identified as a very positive food for a healthy diet and one that also offered convenience.

ii. *Focus groups with consumers who purchase apples in WA to explore their attitudes towards food, healthy eating and the consumption of fresh fruit.*

The first half of the focus groups typically blended discussions of eating habits, apple variety. Participants had no knowledge of the aims of the project during the first discussions. What emerged from the analysis was the key importance of appearance and price, and secondarily flavour and the use of apples within cooking. The high public profile of the Pink Lady™ apple variety was also well illustrated in this diagram, as was the Granny Smith apple variety.

Pink Lady™ was the most commonly known apple variety and emerged within 33% of the transcripts relating to apple knowledge. Other well-known varieties in order of their names appearing in the discussions were Red Delicious, Golden Delicious, Granny Smith and Fuji. Participants commented that the new ANABP 01 apple variety was very "different" to other apples; darker in colour (termed red, or even black). It was commonly noted that it looked like a plum. Upon tasting the vast majority of participants liked the new apple more or on par with Pink Lady™ (Cripps Pink, provided for tasting comparison). Some felt that the new apple has a taste that resembles a green apple, "fresh" and "crisp", others commented that they enjoyed the firmness, whilst some felt that it was "woody", had a "coarse" or "weird" texture; participants were mixed in terms of which variety was more flavoursome. Overall the vast majority of participants would purchase the new apple instead of or in addition to Pink Lady™ or the other apple varieties they purchase, provided that it was of similar price (to the Pink Lady™).

In summary, the themes from focus groups highlighted that in buying an apple consumers are primarily concerned with: price, freshness, flavour, apple variety, colour and cooking/eating preference. The "health" theme did not appear on the concept map, noting that the health benefits of eating an apple did not emerge in any of the focus groups prior to prompting by the facilitator. However, this does not suggest that consumers do not view apples as being a healthy food. It is more likely a reflection that the apple is accepted as a healthy food, as are most fruits. It is also suggested that consumers use colour as a visual means of scoring health quality, as promoted by government and non-government campaigns (e.g. www.healthier.qld.gov.au/about/give-colour-a-spin; www.nutritionaustralia.org/national/resource/eat-rainbow).

iii. *Conjoint experiment via an online consumer panel*

On the basis of the social media surveys and consumer panel, it was decided to use the term "antioxidants" in place of "flavonoids" for the conjoint experiments. While the term antioxidants has particular shortcomings, as acknowledged by the USDA [11], and increasingly by several leading manufacturers, we recognise that the term antioxidant is widely and positively received by consumers and reflects the underlying traits we are seeking to promote. By contrast, consumers have little awareness of the term flavonoid or its meaning.

In the pilot survey, 198 volunteers were provided 16 different combinations of apple characteristics; combinations of colour (four categories), price (four categories), taste (two categories), skin (two categories) and antioxidants (two categories). It was concluded that the "ideal" apple was: red in colour, low in price (<$3/kg), crispy in taste, thick in skin, and high in antioxidants.

Based on the findings of the pilot survey the apple characteristic of colour, taste, and skin were excluded from the survey as they were not significant determinants of a willingness to pay. The “Antioxidants” characteristic was rephrased to “Antioxidants in Apple/ Health Benefit”. New attributes were included to provide contrast to other major relevant consumer trends/ product features on consumer preference; Organic and Origin. A final set of 12 different combinations of attributes and characteristics associated with apples were developed. These were used in the final conjoint study where consumers were asked to consider combinations of the following options:

- Price
- Health Benefit/ Antioxidant content
- Organic
- Origin

Respondents’ demographics, fruit purchasing preferences, apple purchasing and consumption preferences were also surveyed. There were 813 respondents in the final sample. Out of these, 797 were used as these had an adjusted R^2 of 0.30 or higher for the conjoint questions (not filling the conjoint with the same answer throughout). The sample comprised of 54% female and 46% male respondents and covered all age brackets with good representation across these demographics. 60% respondents were professional or semi-professional white collar, skilled trade or self-employed (**Figure 1**). 30% of respondents had a household income >$100,000, with even spread of the remainder down to <$30,000, and >60% respondents were responsible for weekly or daily household shopping. Nearly 80% respondents ate 2-3 apples per week, with 33% eating apples daily (**Figure 2**).

The values shown in **Table 1** provide a measure (in percent) of the relative importance of the single factors for the determination of the utilities. We can see “origin” is the most importance factor accounting for 35.8%, followed by price (30.8%) and antioxidants (24.3%).

In summary, the online survey and conjoint analysis highlighted the primary importance of local origin and price in consumer’s decision to buy apples. Nevertheless, antioxidants were shown to be at least a secondary consideration, and more important to purchase decision than organic.

Table 1. Apple characteristic preferences in order of importance.

	N	Importance score (%)	Std. Deviation
1. origin	738	35.8	19.76
2. price	738	30.8	20.07
3. antioxidants	738	24.3	14.72
4. organic	738	9.0	9.00
R^2	738	0.947	0.05
AdjustedR^2	738	0.794	0.17

Online education of health properties of apples

Recognising the wide variance in consumer knowledge of the health benefits of apples, we established a local (WA) presence to add further information relevant to (i) flavonoids and cardiovascular health, and (ii) apple breeding:
https://www.agric.wa.gov.au/pome-fruit/apples-and-flavonoids
https://www.agric.wa.gov.au/pome-fruit/apple-flavonoids-and-human-health
https://www.agric.wa.gov.au/pome-fruit/breeding-healthier-apples
These pages will be updated as data are prepared for publication from this report.

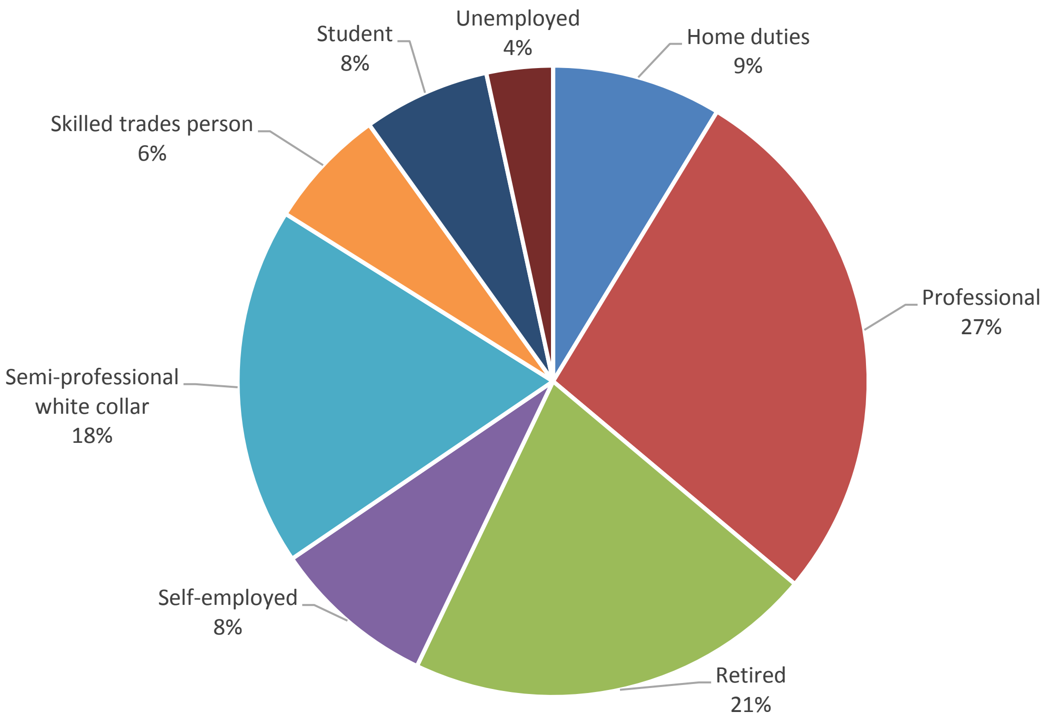

Figure 1. Occupation demographics of respondents to conjoint study.

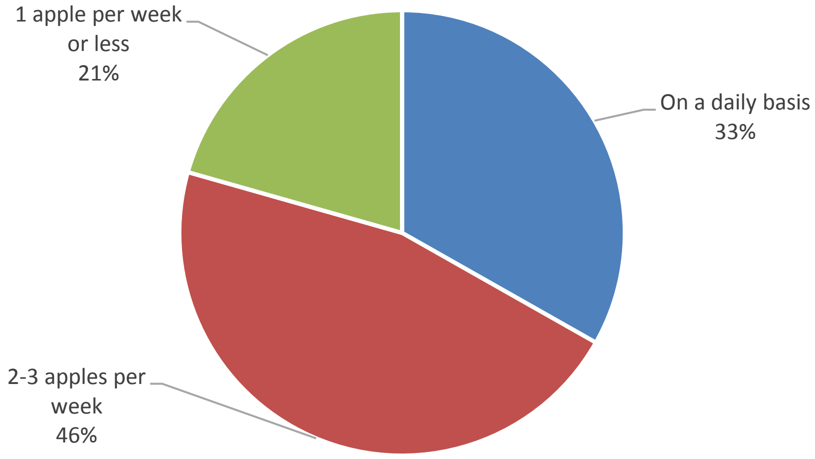

Figure 2. Apple consumption profile of respondents to conjoint study.

Outcomes

The primary outcomes resulting from this project to date have been the development of new collaborations and research proposals, and the increased uptake of these data for promotional activities related to the Bravo™ apple. The significant demonstration of capacity to breed for enhanced levels of flavonoids in apples (Aims 1, 2) has underpinned development of a new project proposal to identify and implement genetic markers for flavonoid content in apples in the ANABP. This proposal also seeks to capitalise on the recent changes to the health claims legislation [7]. The proposal will directly build on the data from this project, and importantly enables the expanded network from WA to the national level and New Zealand.

New collaborations have resulted from the communication of preliminary findings through this project. Important new collaborations include Plant and Food Research for their expertise and resources in rapid-breeding and genetic manipulation of apple, as well as particular progress on enhancing anthocyanin levels in apples. In addition, we anticipate developing direct collaboration with the Queensland Alliance for Agriculture and Food Innovation (QAAFI), Queensland University of Technology and University of Queensland in relation to their compatible research on the Queen Garnet plum. WAAA also have a plum breeding project, with several advanced selections, that could follow directly from the expansion and further funding of this project.

The recently released ANABP 01 (Bravo™) apple has been licensed to Fruit West Co-operative Ltd, who have been working with our research team towards developing minimum standards for quality assurance, based on flavonoid levels. The exact development and implementation of these standards is in progress, and is likely to involve further R&D to develop tailored analytical equipment.

The direct implications and applications of this project also changed somewhat since the project commenced. The revisions to the health claims legislation [7] make an application for a higher level health claim for apples achievable. Our research team have engaged with a legal agency to further communicate the implications of these changes to the broader industry in WA and to hone a strategy to target an application to FSANZ for a higher level health claim, based on the clinical evidence for cardiovascular benefits than this team have provided.

In a longer term strategy, this team are driving towards a national training centre, with a significant node in WA. The training centre would incorporate genetics and breeding for fruit crops, as well as the necessary validation studies and market research to drive opportunities to expand the local and export markets for Australian-bred apples, as well as other significant fruit tree crops. At this stage, the concept is in discussion, however this strategy may not be limited to flavonoids.

Evaluation and discussion

This project aimed to identify and close some of the major knowledge-gaps in the opportunity to selectively breed and market a flavonoid-rich apple. Two particular questions were addressed (refer Introduction). A vast amount of potentially game-changing data and insight have emerged, demonstrating beyond little doubt that:

- Flavonoid composition and content in apples varies qualitatively and quantitatively, by >100-fold in some comparisons.
- Flavonoid composition and content in apples is under a high degree of genetic control, more considerable than environment or cultural influences.
- Particular flavonoids are highly heritable within the population of the ANABP. Particular genotypes were identified with high estimated breeding values for flavonoid content.
- Consumers recognise antioxidant qualities as an important factor influencing purchase decisions, in fact more important than organic status.

Hence this project has significantly closed the gap towards selective breeding of flavonoid-rich apples (question 1) and building the case for added market value (question 2). However, marketing such an apple requires careful planning, particularly with regard to the language used and further education of consumers. Both of these considerations will be greatly advanced by the commitment to seek a higher level health claim for apples under the FSANZ regulation.

The final proof of whether it is possible and profitable to selectively breed and successfully market a new flavonoid-rich apple variety, will only be demonstrated on outcome of the strategies currently being developed for the ANABP. However, several recent examples from other crops provide promise. In addition, the developing experience of the apple industry with the new ANABP 01 (Bravo™) provides a solid test-case. ANABP 01 was found to have exceptional levels of particular flavonoids (data not shown), and the research team are working with industry to develop and use this information strategically.

Breeding flavonoid-rich apples

The means to selectively breed a flavonoid-rich apple will require establishment of some new data and tools. In particular, extending the platform developed here to genome-wide association study (GWAS) for genomic selection. The loss of linkage disequilibrium in apples means that markers for particular traits require a high degree of resolution, up to millions of markers [12]. Nevertheless, genome-wide approaches are now within reach of affordability for the Australian horticultural industries, and evidence of the value of the approach are established in examples of perfectly predictive markers for fruit acidity [13] and fruit firmness and storage life [14]. Technologies for direct cisgenic manipulation (*i.e.* using apple genes) will allow relatively rapid validation of any candidate regulatory genes identified by this method. Further, gene editing technologies such as CRISPR-Cas9 have recently been demonstrated on apples [15], providing potential longer term tools for research and development, even if only at the level of hypothesis or scientific validation. Presently gene editing is governed by the same regulations in Australia as other forms of genetic modification.

In parallel with identifying genomic markers, the pedigree analyses conducted here provide guidance towards strategic crosses using parents with high estimated breeding values for one or more flavonoid. The adoption of rapid-breeding approaches would further accelerate identification of naturally bred but selectively identified new varieties.

Marketing flavonoid-rich apples

Functional foods (foods or their components that may provide benefits beyond basic nutrition) [16] was one of the top five global food trends on Google in 2016 [17], and a healthy perception outweighed convenience and sustainability as one of the most important drivers of purchase for foods and beverages in the USA. As a result, functional foods generated >US$250billion sales in 2014, growing at a compound annual growth rate of 6% [18]. This contrasts with the GVP of apples, US$50-60billion globally and US$400-500million in Australia, growing at a compound rate of 5.5% and 4% respectively (2000-2013) [19].

The experience of the Queen Garnet plum and more recently the Bravo™ apple, together with our data demonstrate that the local market are increasingly aware and selective in their choice of fresh products. In addition, Nutrafruit©, who distribute Queen Garnet also have two products marketed as functional foods; a probiotic powder and a juice. This demonstrates a growing local market, however our data show that local consumers remain heavily driven by price. Clearly there are broader factors of marketing that need to be integrated with the promotional strategy for healthy fruits and vegetables.

To place the values of flavonoids we found in context with other foods and beverages that are widely and successfully marketed on 'antioxidant' quality (see below), **Table 2** provides a brief comparison, based on the range of flavonoids found in the ANABP germplasm.

Table 2. **Comparison of flavonoid content in whole apples represented in the Stage 2 germplasm of ANABP with values from other prominent foods which are widely regarded as healthy**. Values are mg/100g or 100mL. ND = not determined. Data from [20, 21, 22].

	ANABP germplasm	Grape seeds, raw [20]	Green tea [20, 21]	Prune juice [21]	Coffee (Arabica) [21, 22]
Quercetin	31	20	<3	ND	<1
Epicatechin	20	93	8	ND	<1
Chlorogenic acid	56	ND	<3	20	43
Phloridzin	>4	ND	ND	6	ND
Anthocyanin	40*	ND	ND	ND	ND

* Most genotypes <10 mg/100g

The case for a higher level health claim

The contribution of fruits to the diet is one of the top three predictors of disease and morbidity associated with non-communicable diseases [23]. Flavonoids have emerged as one of the more important non-nutrient components of fruits and vegetables [2, 3]. Dietary flavonoids contribute to a variety of activities that can positively influence blood pressure, inflammation, glucose metabolism and a number of other cardiovascular, anti-cancer and cognitive functions. Indeed, our research team have demonstrated significant benefits in cardiovascular function within hours [24] of eating a flavonoid-rich apple meal, and recently substantiated these benefits in a four week trial [25]. Additional work has demonstrated benefits in cognitive function [26]. Importantly, these were the result of trials with an achievable intake of apples, providing as little as *ca*. 180, 50, 5, 50 and 6mg per day quercetin, epicatechin, phloridzin, chlorogenic acid and anthocyanin respectively [24-26]. And further supported by a prospective study based on elective consumption [27]. However, an important remaining question is how much is enough? The study designs [24-26] incorporated a control, low flavonoid apple, which still contained >5mg quercetin and epicatechin, and up to 50mg chlorogenic acid. This question requires further attention.

Nevertheless, these data strongly support and are perhaps sufficient to warrant application to FSANZ for a higher level health claim for apples. The view of the research team is that this be made on a national basis for all fresh apples, with varietal differentiation provided through minimum maturity standards and content claims, rather than targeting a higher level health claim for individual varieties.

Challenges for marketable terminologies

While the advertising of some functional foods is based on approved health claims (*e.g*. folate), others are driven by marketing trends. With a healthful perception being among the leading drivers of consumer purchasing decisions in the USA [30], there has been an expansion in marketing terms, such as 'antioxidant' and 'superfood', now synonymous with a perception of 'healthy'. This reality was reinforced by our social media survey in relation to apples. Nevertheless, government agencies and leading food manufacturers are now distancing their products from these ill-defined terms.

The term antioxidant is particularly problematic [28, 29]. Flavonoids are not nutrients or vitamins but can generate additional health-promoting properties. For this reason flavonoids have variously been termed antioxidants, phytochemicals or phytonutrients, in addition with other classes of plant chemicals such as glucosinolates. In nutritional research, the term was originally used in context of the 'free radical theory of aging' [31] and later applied as a lay term for phytochemicals. Popular support for the theory saw development of antioxidant activity assays and resulted in large public datasets of the antioxidant values of foods, such as that led by the USDA [11]. However, a number of meta-analyses of clinical studies have failed to support the theory and recent studies in fact found supplementation increased mortality (albeit not with flavonoids) [29]. For this reason the USDA withdrew the antioxidant database [11]. Researchers now agree that the benefits of phytochemicals come most often through additive and synergistic benefits in the whole foods, and differentiated from antioxidant activities [32]. Our research team encourage further discussion at the national level to consider the pitfalls of allowing terms such as antioxidant to proliferate. More specifically to apple, we encourage the national body to revise the Aussie Apples website.

Conclusions

This project has unequivocally demonstrated the capacity to breed flavonoid-rich apples, building on data from associated studies that demonstrate significant benefits to dietary health. Additionally, it has demonstrated a significant opportunity to capture value from marketing the healthy qualities of fruits, which we found was more positively accepted by consumers than organic status. This finding alone should give confidence to fruit breeders and marketers to expand health-related RD&E, but with several cautions. In particular, the research team believes a careful debate be had on the appropriate terminology for use in marketing and that industries carefully study the revisions to legislation governing health claims and nutrient content claims. Similarly, we believe that a validated health claim for apples is an achievable goal in the near term, but suggest varietal differentiation be made on set maturity standards, content claims, and strategic marketing and consumer education to underpin greater appreciation of the healthy values of fresh Australian horticultural foods. The research team have concluded a number of targeted recommendations that should steer the fresh fruit and vegetable industries towards capturing some of the value of existing and new varieties.

Recommendations

1. Support a national application for a higher level health claim for apples relating to cardiovascular disease (not variety specific).
2. Hold a national debate of the use of specific terms for promoting phytochemical-derived benefits in fresh fruits and vegetables.
3. Support R&D directly following from this project to establish marker-assisted, rapid breeding technology in the Australian National Apple Breeding Program, initially targeting flavonoid content.
4. Support further R&D to validate and extend dietary health outcomes from apple consumption to major non-communicable diseases.
5. Support a national training centre to develop expertise and personnel to drive new biotechnologies in the perennial fruit industries.

Scientific refereed publications

<u>Journal article</u>

Bondonno, C.P., Bondonno, N.P., Considine, M.J., Shinde, S., Swinny, E., Jacob, S.R., Lacey, K., Croft, K.D., Hodgson, J.M., 2017 (Submitted). Phenolic composition of 91 Australian apple varieties: identification of Australian-bred apples with enhanced health attributes. Food Chemistry.

<u>Chapter in a book or Paper in conference proceedings</u>

Seaton, K., Considine M.J., 2017 (in press). Maximising the levels of dietary flavonoids in apples and Rosaceae. In: Pareek, S. (ed.), Novel Postharvest Treatments of Fresh Produce (A volume in series 'Innovations in Postharvest

Technology'). CRC Press, Taylor and Francis Group, Boca Raton, FL, USA. ISBN: 9781498729918.

Shan, F., Considine, M.J., Bondonno C.P., Seaton K., 2017 (in press). Flavonoids in pome fruits - health benefits, genetic control and prospects to breed an even healthier apple. In: Pareek, S. (ed.), Novel Postharvest Treatments of Fresh Produce (A volume in series 'Innovations in Postharvest Technology'). CRC Press, Taylor and Francis Group, Boca Raton, FL, USA. ISBN: 9781498729918.

Nelson MN, Cowling W, Bondonno CP, Bondonno NP, Croft KD, Hodgson JM, Lacey K, Jacob S, Considine MJ (2017) Towards pedigree analysis of dietary flavonoids in apples. In: 9th International Workshop on Anthocyanins, Auckland, New Zealand, February 2017.

Cowling W, Nelson MN, Bondonno CP, Bondonno NP, Lacey K, Jacob S, Croft KD, Hodgson JM, Considine MJ (2017) Heritability analysis of dietary flavonoids in apples. In: N8 Agrifood 2017 International Sustainable Food Production Conference, Durham UK, July 2017.

Cowling W, Nelson MN, Bondonno CP, Bondonno NP, Lacey K, Jacob S, Croft KD, Hodgson JM, Considine MJ (2017) Heritability analysis of dietary flavonoids in apples. Fourth International Horticulture Research Conference, East Malling UK, July 2017.

Intellectual property/commercialisation

The data generated by this report lend towards new commercially valuable intellectual property. For this reason, sensitive data are reported in a Confidential Appendix and cryptically coded, so as to preserve the potential until industry can develop a strategy to capture it.

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Contributors

Michael Considine led the research and wrote the Final Report. Fucheng Shan, Kevin Seaton (WAAA) contributed to the desktop studies for Aim 1. Catherine Bondonno (UWA, ECU), Nicola Bondonno (UWA), Jonathan Hodgson (ECU) and Kevin Croft (UWA) led the flavonoid analysis (Aim 2), with critical input from Steele Jacob, Kevin Lacey, John Sutton and Diana Fisher (WAAA). Matthew Nelson (Kew Gardens) and Wallace Cowling (UWA) led the genetic and pedigree analysis (Aim 2). Tim Mazzarol, Elena Limnios and Geoff Soutar conducted the market research (Aim 3). Alison Matthews and Rachelle Johnstone (WAAA) created the online pages of the health qualities in apples (Aim 3).

Appendices

AP12036 Confidential Appendix