Key genes for horticultural markets

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Know-how for Horticulture™

FINAL REPORT

HORTICULTURE AUSTRALIA Ltd

PROJECT AH01015 (31st March 2007)

KEY GENES FOR HORTICULTURAL MARKETS

Steve Swain *et al.*

CSIRO PLANT INDUSTRY

HAL project number: AH01015 (31st March 2007)

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MEDIA SUMMARY

The Key Genes for Horticultural Markets program was established to characterise plant genes with potential to improve horticultural crops and increase Australia's competitive position in world markets. Targeted traits included the consistency, availability, and quality of fruit and vegetables in the marketplace and enhanced novelty in terms of size, shape, colour, 'mouth feel' properties and antioxidant content.

The program was comprised of three interlinked approaches that generated gene-based tools for use in horticultural breeding programs:

- Approach one focused on a gene, named *ARF8*, which is central to seed set and to the shape and size of both seedy and seedless fruit. This research identified critical components of reproductive physiology that limit fruit set, and has provided important information for the development of new seedless varieties for a range of horticultural crops. Proof-of-concept experiments in the model crop tomato demonstrated that modified forms of the *ARF8* gene can be used to enhance seedless fruit production.

- In approach two, novel genes to facilitate the action of the plant growth regulator gibberellic acid and enable the manipulation of stem, leaf, fruit and seed characters have been identified. This research identified two groups of candidate genes: auxin related genes that can be used to promote stem and fruit growth and genes that regulate the action of gibberellic acid and enable seedless fruit production.

- The third approach built on the first two and identified genes that modify the important characters of colour, mouth feel and health properties. The main focus was on two related classes of plant chemicals that are involved in the determination and expression of leaf and fruit colour (anthocyanins), and in flavour and mouth feel (tannins). The research characterised a class of genes (known as the *PAP* genes) that were shown to induce colour formation in leaves, seeds and fruits of test plants. Related work in apple demonstrated that skin colour (green or red) is controlled by an apple *PAP* gene. Based on this discovery, a gene-based marker was developed that can be used in breeding programs to accurately predict skin colour in apple seedlings many years before flowering occurs.

The crucial next step will be to ensure that Australian horticulture benefits from this research by using these tools to improve the efficiency of delivering superior new varieties through major Australian breeding programs.

TECHNICAL SUMMARY

World-wide advances in bioscience are opening up new opportunities to increase the success rate for developing exciting new fruit varieties that meet and exceed consumer expectations. Australia's competitors are clearly embracing these technologies and, for example, are using gene-based tools in horticultural breeding programs. In addition, large gene sequencing projects that will greatly accelerate the development of these tools are already underway in various countries for horticultural crops such as citrus, grapes and apples.

Conventional, non-GM, breeding for Australia's horticultural industries fundamentally involves re-combining useful genes, which control key traits, into a single plant that can then be propagated either by seeds or vegetatively. Gene-based tools such as molecular markers can be used to increase the efficiency of this process. Australia's competitors, including the USA, China, Spain and Japan, are making significant investments in genomics and the use of molecular markers for conventional breeding, and are ahead of Australia in the use of this technology.

To ensure Australia's competitive position in this bioscience-driven new era, the Aushort Key Genes for Horticultural Markets project was established by HAL in 2001. This project focused on identifying, isolating and characterising new genes that can be used to increase the consistency of quality and availability of fruit and vegetables in the marketplace and to enhance their novelty in terms of size, shape, colour, 'mouth feel' properties and antioxidant content. The strategy was to identify target genes that can be used to alter these characteristics through conventional breeding, contributing to the future growth of Australian horticultural products in domestic and export markets.

This "high science" gene discovery project was conducted at CSIRO by Dr Steve Swain, Dr Mandy Walker, Dr Simon Robinson and Prof. Anna Koltunow using a three-stranded approach. The main output of the project has been the delivery of tools that enhance the ability to select accurately for important fruit traits. These are seedlessness and fruit pigmentation due to anthocyanins, which are responsible for the red colour and health benefits of many fruit crops.

Approaches 1 and 2 have identified genes that control the action of two plant hormones, auxin and gibberellic acid (GA), that are commonly used to modify fruit growth in a number of horticultural crops. In proof-of-concept experiments an auxin gene (known as ARF8) has been used to enhance the production of seedless fruit in tomato, while in Arabidopsis both the auxin and GA genes have been successfully used to generate seedless fruit. In these model plants, as for horticultural crops such as citrus and grapes, application of auxin and/or GA can also lead to seedless fruit production – in other words auxin and GA genes have been used to replace chemical application of these plant hormones. In terms of conventional breeding programs in which seedless fruit production is a key trait, including citrus, table and dried grapes, this knowledge could be used to develop gene based markers. Markers can greatly improve the efficiency of existing breeding programs by allowing selection of the best, i.e. seedless, progeny many years before fruit production occurs. Selection at the young seedling stage can ensure that only seedless progeny are evaluated in the field, increasing the likelihood of the successful development of new elite varieties.

Approach 3 focused on flavonoids such as anthocyanin, which are involved in fruit colour and antioxidant levels, and tannins, which contribute to flavour and mouth feel. This research identified genes that regulate the anthocyanin/tannin synthesis pathway in the model plant Arabidopsis, to enable the isolation of the appropriate genes from crop plants to alter colour, mouth-feel and antioxidant levels. In fact, a parallel project funded by the Department of Agriculture and Food, WA has built on the *Arabidopsis* work to identify the gene that controls

colour, i.e. red vs green skin, in apple. Using this gene, a marker has been developed that enables apple skin colour to be predicted at the young seedling stage. While this represents a significant advance in the use of gene-based markers in apple breeding, and could be used to improve breeding efficiency as described above, it also provides the ability to develop more sophisticated breeding tools in the future. For example, with additional research it should also be possible to develop a marker for early selection of seedlings that will produce a pink hue similar to the highly successful Pink Lady™ apple, allowing breeding programs to target particular types of skin colour.

The crucial next step will be to ensure that Australian horticulture benefits from this research by using these tools to improve the efficiency of delivering superior new varieties through major Australian breeding programs.

INTRODUCTION

CSIRO Plant Industry originally presented a proposal to Horticulture Australia Ltd (HAL) in the area of plant molecular genetics and physiology, particularly in the growth, form and colour development of fruit, seeds and leaves. This proposal was selected through a competitive tender process and was the first, and to date only, strategic gene discovery initiative funded by HAL.

The proposal consisted of strategic research on the identification of key regulatory genes involved in plant development which could subsequently be applied to a range of different crops, maximising the return on investment in research and development to Australian horticultural industries.

The research aimed to provide genes with a generic usefulness in plant improvement for the benefit of both consumers and producers and to demonstrate, in appropriate model plant systems, that these genes will function predictably to provide the stated desirable trait.

The delivery of outputs in this proposal was underpinned by:

- focus on areas of plant development and physiology that were already a part of the core scientific discipline of CSIRO Plant Industry
- participation of four scientists who are leaders in the fields selected for strategic research in this proposal
- the associated body of scientific expertise and personnel working in closely related areas across the agronomic and molecular programs of CSIRO Plant Industry
- a track record of patenting and managing of intellectual property to maximise its intrinsic value and return to industry
- an extensive CSIRO-developed technology package which is accessible to Australian industries and companies
- strategic alliances with multinational companies to access enabling technologies, to aid in product development
- linkage to a range of national horticultural breeding programs in fruit, nut and vegetable crops

Arabidopsis thaliana

The research was focused on three interlinked Approaches. The model laboratory plant *Arabidopsis thaliana* was used as the major genetic background to isolate useful genes. Among its advantages are its very short generation time and that the entire genetic sequence of this plant had recently been determined. This provided a gene bank of sequences that were freely available together with a range of traditional, genetic and molecular tools to examine gene function during developmental processes. In the case of Approach 1 tomato provided an additional plant within which effects on fruit growth were monitored because of its short generation time and existing range of established quality parameters to compare benchmark changes. In Approach 3 tobacco and *Matthiola incana* were also used as model plants because flower colour has been extensively studied in these species. A component of this Approach involved direct research on skin colour in apples. This component was partly supported by the Department of Agriculture and Food, Western Australia.

Modulators of yield, shape, texture and seed content (Approach 1)

Significant losses of quality and yield can occur in horticultural crops because of poor fruit and seed set. Optimisation of fruit and seed set for predictable yield of high quality is of

paramount importance to producers, while consumers place greatest value on reliable product appeal, taste and price. In preliminary work funded as part of the Citrus scion breeding project, CSIRO isolated a gene called *ARF8* from the model plant Arabidopsis that appears to be involved in regulating fruit and seed set (Figure 1). This component of the research aimed to establish its use in controlling both fruit and seed set in Arabidopsis and tomato. Initial studies of this gene in Arabidopsis suggested that, with the addition of other interacting factors, it may enable alterations in fruit shape and texture and provide the flexibility of retaining or eliminating seeds from a variety of crops.

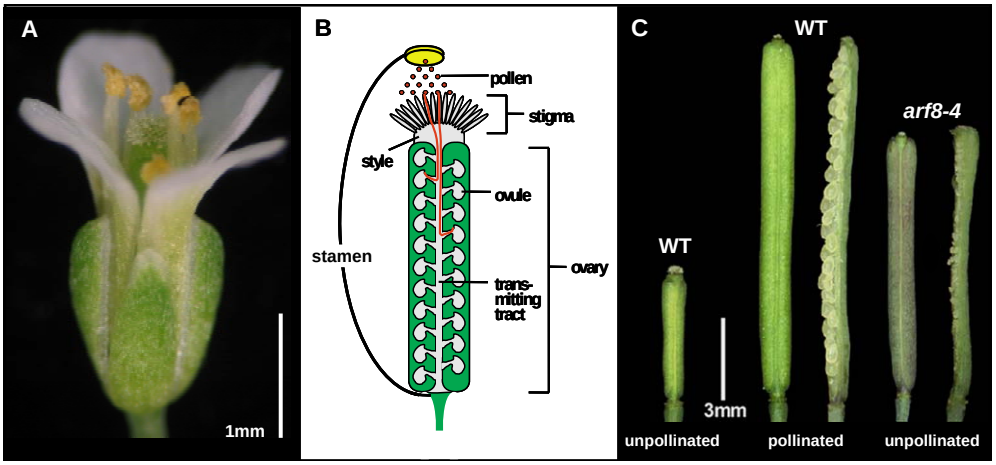

Figure 1. Fertilization and seed set are usually essential for fruit initiation.

A. Photo of an open Arabidopsis flower at anthesis (pollen shedding). The sepals (green) partly enclose the petals (white), the stamens (yellow anthers at the top), and the central gynoecium consisting of the stigma, style and ovary. The anthers have just begun to deposit pollen onto the stigma. **B.** Schematic diagram of a WT Arabidopsis flower with sepals and petals removed. In this essentially self-pollinating species, pollen grains produced by the anther germinate on the stigma to produce pollen tubes (shown in red) containing two sperm cells that grow through the transmitting tract towards the ovules. Once a pollen tube reaches an ovule it enters inside and into the female gametophyte where it releases the sperm cells. Double fertilization occurs as one sperm cell fuses with the egg cell to initiate embryo formation and the other fuses with the central cell nuclei to initiate endosperm development. At this point seed set occurs and as embryo and endosperm development proceed the surrounding ovule tissues differentiate into a seed coat. **C.** ARF8 prevents fruit initiation in the absence of seed set. The gynoecium of unpollinated WT flowers (*left*), in which no seed set occurs, exhibit little growth beyond the stage shown in (A). Pollination and seed set promote fruit initiation and subsequent growth (*center*). By contrast, loss-of-function *arf8* alleles allow fruit initiation and growth in the absence of seed set (*right*).

GA regulation of plant growth and form (Approach 2)

The plant growth regulators, gibberellins (GAs), are used extensively on a range of agricultural and horticultural crops. GA application to Sultana grapes increases berry size and in citrus fruit it maintains rind quality and reduces the incidence of fruit disorders. In other crops GA biosynthesis inhibitors and genotypes with reduced GA activity are used to control vegetative growth and size. In preliminary research, a gene encoding an enzyme that degrades GAs was introduced into Arabidopsis to cause altered vegetative growth and fruit size. These plants were used as a genetic background for a mutagenesis screen to isolate new GA-related genes. The eventual aim was to use these genes either individually, or together with other genes isolated in this proposal (in Approaches 1 and 3) to manipulate the growth and size of different plant organs, including vegetative, seed and fruit structures in horticultural crops. During the course of this project knowledge of how plants perceive and respond to GA

greatly increased (Figure 2), allowing the analysis of fruit growth in GA-related mutants potentially able to produce seedless fruit.

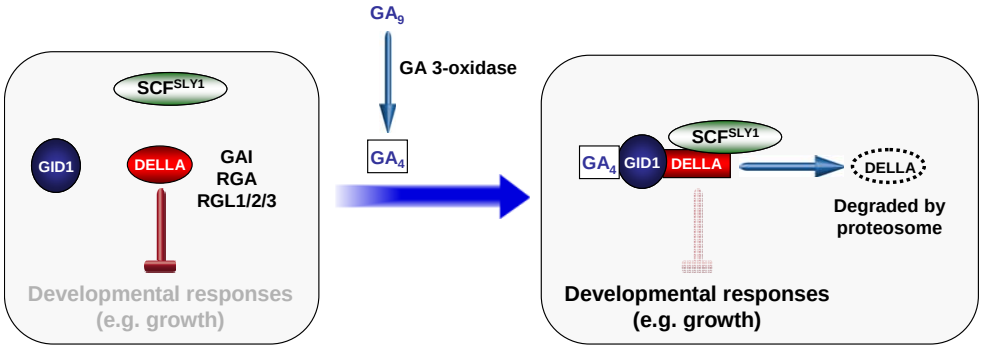

Figure 2. GA action is mediated by the degradation of DELLA proteins.

In the absence of GA the DELLA proteins inhibit processes such as leaf and fruit growth. Active GAs, such as GA_4 in Arabidopsis, are perceived within cells by the GA receptor (GID1). This causes degradation of the DELLA proteins and allows growth to occur. Similar genes are present in all horticultural crops examined to date, including grapes and citrus.

Modulation of flavonoid biosynthesis to alter tannin, colour & health properties (Approach 3)

The two approaches described above concentrated on the quality and reliability of the product. This part of the proposal explored the possibility of directly modifying other factors by which consumers judge value. These included the end products of the flavonoid biosynthesis pathways in plants, particularly anthocyanins, which provide colour and antioxidant properties to food, and tannins, which contribute to the astringency or 'mouth-feel' quality of fruits and vegetables (Figure 3). Too much tannin can lead to unpleasant mouth-feel and problems in processing. While three components regulating the late part of the anthocyanin pathway had been identified, critical factor(s) regulating anthocyanin accumulation and the branch to the tannin biosynthetic pathway were needed to develop this approach. Most plants can make some anthocyanin in selected tissues including leaves, flowers and fruit, or under specific conditions such as stress or high levels of UV. This suggested that all the essential components of the flavonoid pathway are present within the genome of the plant. By identifying and understanding the relevant regulatory factors required for expression of the anthocyanin genes in the late part of the pathway in the model plants Arabidopsis and Matthiola, we aimed to determine which factors are important in, or absent from, tissues giving rise to fruit and vegetables. This would provide the potential to control this pathway to produce a range of horticultural crops with novel appearance and improved health benefits. A crop for direct application was apple, and a closely linked project was developed to characterize apple genes involved in anthocyanin/tannin production in detail.

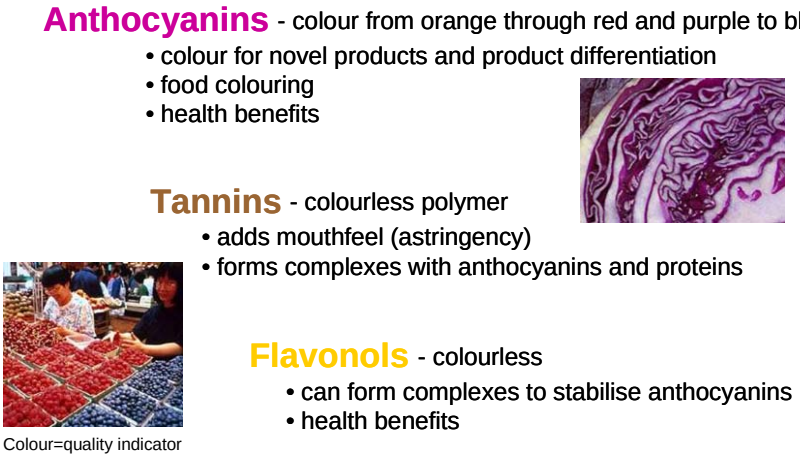

Figure 3. Flavonoids are important for consumer appeal in a range of horticultural crops.

Materials & Methods

A three stranded approach for this proposal was developed based upon and linking discoveries and skills of the key researchers.

Approach 1 built upon our knowledge of a gene, *ARF8* (originally *FWF*), shown to modulate the initiation of fruit growth, fruit shape and seed content in Arabidopsis. The application and utility of *ARF8* was assessed in plants by understanding its mode of action at the molecular level and proof-of-concept experiments in tomato.

Approach 2 focused on the *2ox2* gene in transgenic Arabidopsis, known to be involved in the deactivation of gibberellin, a phytohormone that regulates plant growth. Alterations in the activity of the 2ox2 gene resulted in novel effects on fruit and seed development. Plants expressing this gene were used as a genetic background to isolate other genes with potentially useful effects in plant growth. Mutants with defects in other GA-related genes were also examined for their effects on fruit growth.

Approach 3 aimed to identify the missing elements that regulate the anthocyanin/tannin synthesis pathway in Arabidopsis. This project focused on the PAP genes which were characterized in detail and tested for their ability to manipulate anthocyanin/tannin production.

Approach 3a identified apple genes involved in anthocyanin/tannin synthesis. The effect of environmental conditions (light vs dark) known to influence apple skin colour was then shown to control expression of these genes. Finally, the molecular basis of several green-skinned apple varieties was shown to be caused by changes in an apple *PAP*-like gene.

Activities 1-34 have been described in previous milestone reports. Each milestone was comprised of several activities reported in detail to HAL throughout the course of the project. Activities were a natural progression, with outputs of each feeding into the next activity within each approach, such that only activities 35 to 40 are reported in detail in this report. Due to the number and size of the activity reports, it was not possible to include them all with this report".

All molecular research was conducted in OGTR-approved facilities.

RESULTS

Final report for Approach 1

Activity 36: *Analyze ARF8:Myc lines and create and analyze 35S:ARF8:Myc lines to determine protein sizes of ARF8 and arf8-4 by Nov 30th 2006.*

Details of this activity:

We failed to determine the size of the protein products due to problems with the antibody in terms of cross reaction. We also tried to analyze the size of the protein in ARF8:GUS transgenics but the commercial antibody had a very low titre. Such problems are not uncommon in this type of research.

We focused instead on the characterization of the tomato *ARF8* gene and demonstrated that it produces a splice variant which might contribute to the background parthenocarpy observed in most tomato cultivars. Marc Goetz, the postdoc working on this project, ceased on Dec 6, 2006. Anna Koltunow, Susan Johnson and Julio Rodruiges at CSIRO have continued to finalize experiments concerning the expression of the variant in tomatoes and the quantification of parthenocarpy in plants containing the variant transcript. Collectively all of the work to date shows that *ARF8* regulates parthenocarpy in tomato and that most tomatoes contain the splice variant and most have a low degree of background parthenocarpy. This result is consistent with our observation that the addition of competing mutant Arabidopsis *arf8-4* transcript to the Monalbo variety increases the extent of parthenocarpic fruit initiation and size of fruits (Figure 4).

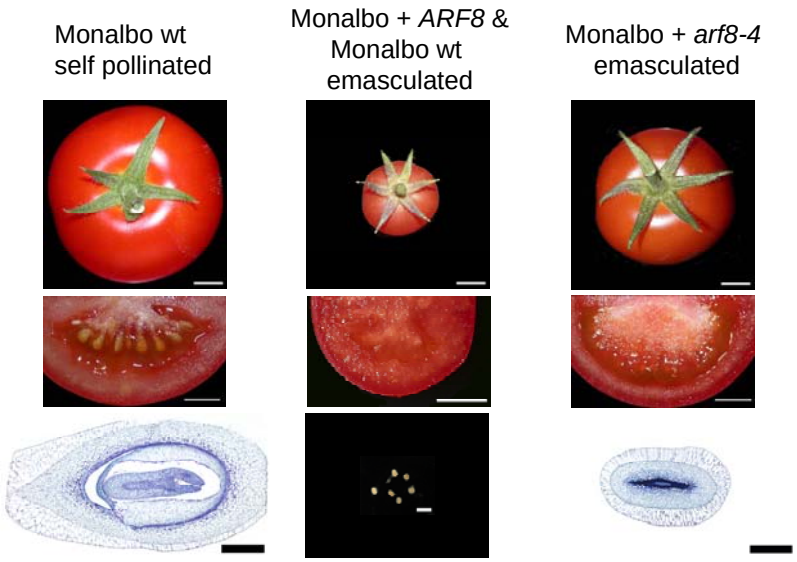

Figure 4. *arf8-4* enhances parthenocarpy in tomato.

Many tomato varieties, including Monalbo, display limited background parthenocarpy likely due to an existing mutation that affects *ARF8* mRNA production. Addition of the mutated Arabidopsis gene, *arf8-4*, enhances this background parthenocarpy and increases the size of seedless fruit following emasculation.

Final report for Approach 2

Activity 37: *Isolate plants lacking functional DELLA genes that also have impaired ARF8 function by Nov 30th 2006.*

Details of this activity:

Previous work with the della mutant suggested that enhanced GA response stimulates fruit development in the absence of pollination and fertilization. Since a similar phenotype is observed in loss-of-function *arf8* mutants, we attempted to examine the interaction between the two types of mutant. Because the della plants have poor fertility and carry mutations in 4 different genes encoding DELLA proteins, making the pentuple mutant with *arf8-4* would be difficult. Instead we supertransformed the della mutant with a dominant-negative mutant form of ARF8, pARF8:arf8-c-myc. After two transformation attempts, only two lines transgenic for the pARF8:arf8-c-myc transgene were obtained. This may be due to the reduced fertility of the della mutant which is further exacerbated by reduced ARF8 function. Emasculation of flowers from these two lines did not produce larger parthenocarpic fruit than was observed in the original della mutant (Figure 5). Given the difficulty in recovering transgenic lines in this background, it is possible that these lines possess sufficient residual activity of ARF8 to inhibit fruit growth, and a stronger reduction in ARF8 activity would result in larger seedless fruit.

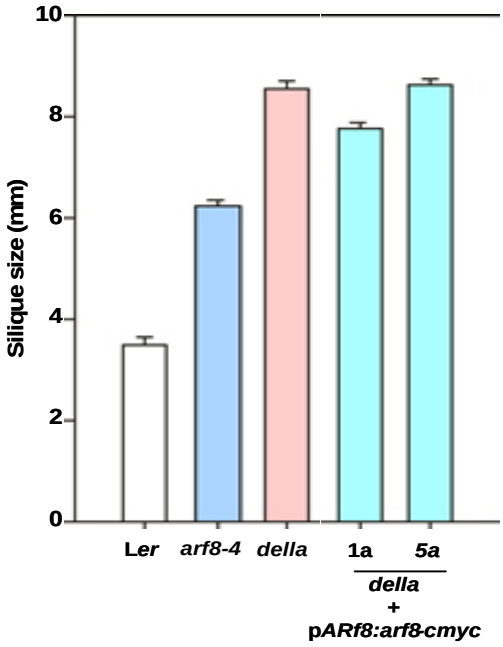

Figure 5. Seedless fruit size in plants lacking 4 of the 5 DELLA genes is not enhanced in two independent lines carrying the pARF8:arf8-c-myc transgene.

Activity 38: *Complete analysis of parthenocarpy in plants lacking four of the DELLA genes, including the role of the anthers, by Sept 30th 2006.*

Details of this activity:

Previous work in Arabidopsis with *ARF8* suggested that the pollen has an inhibitory role in fruit initiation and growth that influences the degree of parthenocarpy obtained in plants lacking *arf8*. In contrast, the parthenocarpy exhibited by plants lacking 4 of the 5 DELLA genes appears to be independent of the pollen (Figure 6). This result suggests that the DELLA proteins inhibit fruit initiation and growth by acting downstream of ARF8 and the pollen inhibitory signal(s). This is an important result because it suggests that parthenocarpy due to increased GA action does not depend on physical removal of the anthers.

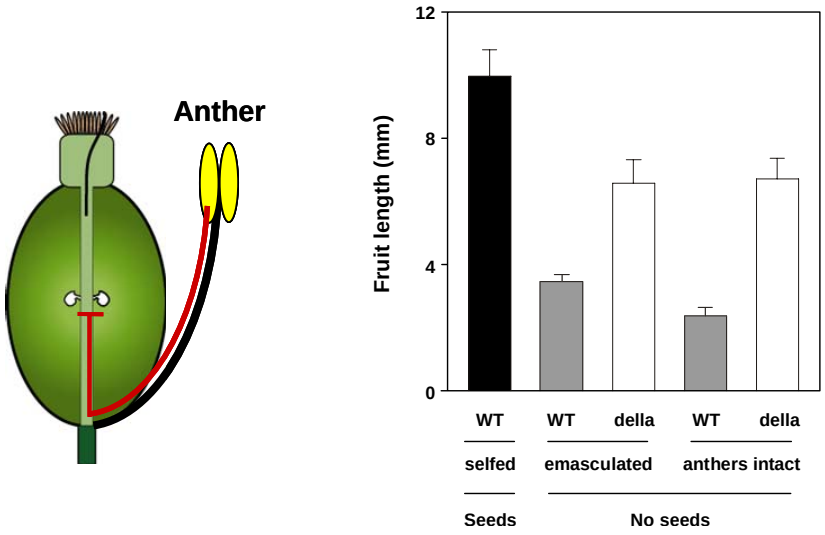

Figure 6. Arabidopsis plants lacking DELLA genes are parthenocarpic.

Left: Schematic model of a fruit just before fertilization indicating that an unknown signal generated by pollen inhibits fruit initiation. *Right*: Fruit (silique) growth in seeded WT fruit compared with seedless fruit on plants lacking 4 of the 5 DELLA genes with anthers intact or removed (emasculated).

Final report for Approach 3

Activity 39: *Determine what contributes to the effectiveness of the PAP1 and PAP4 proteins as regulators of anthocyanin by Mar 13th 2007.*

Details of this activity:

Initial studies suggested that a PAP4 construct in Arabidopsis resulted in much more anthocyanin in the plant than a similar construct containing the PAP1 gene. This difference could be due to either variation in the relative ability of the proteins to control anthocyanin biosynthesis or to differences between the constructs themselves. The PAP4 construct contained an intron as this was the cDNA recovered initially for this gene. The presence of introns in gene sequences used for transformation has been shown by others to increase the relative activity of transgenes, possibly by stabilizing the mRNA as it is being exported from the nucleus.

Constructs for the constitutive expression of PAP1-4 were made with either the genomic sequence with the two introns present or just the coding region without introns. All eight constructs were tested in Arabidopsis by stable transformation. Constructs containing the introns produced plants with more anthocyanin than those with the coding region only. PAP1, PAP2 and PAP4 produced a similar phenotype while PAP3 had a relatively weak phenotype. The coding region constructs result in plants with red seeds and the siliques. The plants with the genomic constructs (i.e. with introns) had a strong purple phenotype in most tissue as shown in Figure 7.

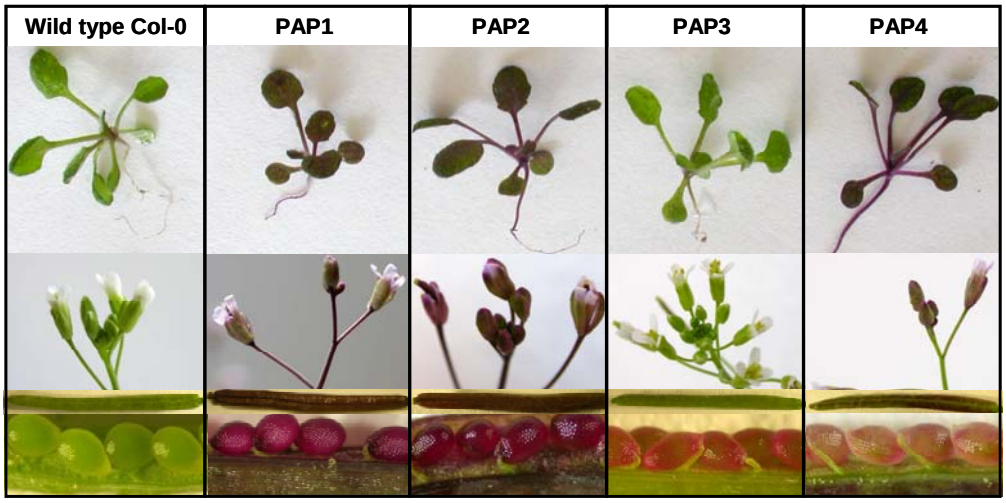

Figure 7. Phenotype of Arabidopsis plants constitutively expressing genomic constructs of the PAP genes.

By contrast, the leaves, stems and flowers of transgenic plants with constructs lacking introns resembled Columbia wildtype plants.

An interesting observation is that the seed phenotype in the PAP4 genomic construct plants is weaker than the phenotype produced by another PAP4 construct that contained only intron 2. Based on this observation, an additional construct containing PAP4 with just intron 1 was made and used to transform Arabidopsis. This new construct was not as effective a regulator as the construct containing intron 2 suggesting that not all introns are equal in effectiveness.

Differences in the intron position in the gene sequence, the intron sequence, or length of the intron might all contribute to the effectiveness of different introns.

These results clearly demonstrate that for the effective design of gene constructs for overexpression of anthocyanin regulators, and probably for other types of genes as well, it may be important to include intron sequences.

Further experiments were conducted to pinpoint the differences between the tannin and anthocyanin regulators of the pathway. Domain swap and mutagenesis experiments have shown that there is a critical region that helps to define the specificity. This finding is novel as the region has not been identified by other groups working in this field.

Activity 35: *Initiate experiments to characterize the MdMYB1 promoter sequence to identify polymorphisms which have functional significance by Dec 28th 2006.*

Details of this activity:

Several differences were found in the promoters of the alleles conferring either red or green apple skins, and one of these differences was used to successfully design a CAPS marker for predicting skin colour.

Polymorphisms were found in two promoter elements predicted to bind particular types of transcriptional regulators that could potentially control MdMYB1 expression. Experiments were initiated to determine if these polymorphisms contribute to the difference between activation of MdMYB1 in red skin versus green skin. This type of experiment is often difficult and can require a great deal of effort and luck to be successful. Although only a short time was available, it was judged to be worthwhile to try this approach. Several attempts were made to isolate and identify CBF-type transcription factors as in other species such as Arabidopsis this type of protein binds to the consensus sequences found in the apple MdMYB1 promoter. Methods to assess the promoter sequences in transient assays and using plant transformation techniques were also investigated but a suitable system could not be established in the available time-frame.

Final report for Approach 3a

Activity 40: *Initiate experiments to characterize other apple MYBs which are expressed in apple fruit skin and which may also be regulators of the flavonoid pathway by Dec 28th 2006.*

Details of this activity:

During the initial work on the isolation of *MdMYB1*, several other MYB genes were isolated from apple based on their sequence homology, searching unannotated database entries and examining the MYB sequences entered by Hortresearch, NZ.

One MYB (MdMYB2) has a very similar R2R3 repeat region to MdMYB1, but the C-terminal region is quite different. An unusual motif was found within this region and identified by homology searches as an EAR motif. This type of motif has been previously identified in transcriptional repressors including the strawberry gene FaMYB1 which is expressed before the fruit starts to colour and prevents transcription of the anthocyanin pathway genes.

To test if the apple gene MdMYB2 could act in the same way, tobacco was transformed with a construct containing the gene driven by the constitutive 35S promoter. Plants containing the transgene were normal until they flowered, when it was clear that anthocyanin synthesis had been inhibited. The flowers of several of the transgenic plants were white instead of the usual mid pink colour of tobacco (Figure 8). Thus it is likely that MdMYB2 is a negative regulator of anthocyanin production.

Figure 8. Overexpression of MdMYB2 in tobacco leads to white flowers

A fragment of MdMYB4 was found in a similar way and the sequence was extended by identifying sequences in the EST database. This sequence is most similar to a tobacco gene which responds to wounding in the plant. The tobacco gene then up regulates the PAL gene which encodes an enzyme responsible for making substrates for the flavonoid pathway amongst other pathways. To enable testing of the function of the apple MdMYB4 gene, the promoters of the PAL genes from apple were isolated and ligated to the luciferase reporter gene and these could in a future project be assayed in the grape suspension cells to determine if MdMYB4 can regulate any of the apple PAL genes. This would also help establish the roles of the four apple PAL genes previously isolated as part of an honours project associated with Approach 3a.

Two other genes identified were similar to a tannin regulator from grapevine. When these genes were used in a transient assay with the ANR promoter, one of the genes was able to switch on the test promoter suggesting a role in the regulation of tannin synthesis.

This aspect of the project has provided some promising areas of research that could potentially be pursued in a future project on apples.

DISCUSSION

The Aushort Key Genes for Horticultural Markets project has delivered significant discoveries and intellectual property of value to a wide range of horticultural crops across two main areas.

Fruit initiation and growth

The focus of Approach 1 was the *ARF8* gene, which encodes an auxin signaling component that acts as a negative regulator of fruit initiation in Arabidopsis. Based on the existence of *ARF8*-like genes in crop plants, and the discovery that cultivated tomatoes produce an altered form of the *ARF8* mRNA which may contribute to limited parthenocarpy (see Results section above), this function is predicted to be conserved across all horticultural crop plants.

A conserved role for an auxin/ARF8 pathway in fruit initiation is further supported by Wang et al. (2005). These researchers used tomato as a model system to investigate the role of a specific Aux/IAA protein, IAA9, using an antisense (AS) gene silencing approach to specifically reduce *IAA9* expression. Aux/IAA proteins can form protein complexes with ARF proteins (Figure 9), and AS-*IAA9* plants initiated fruit development before fertilization, giving rise to seedless, parthenocarpic fruit in a high proportion of flowers. This phenotype suggests that IAA9 normally functions to prevent premature fruit initiation in the absence of fertilization and seed set.

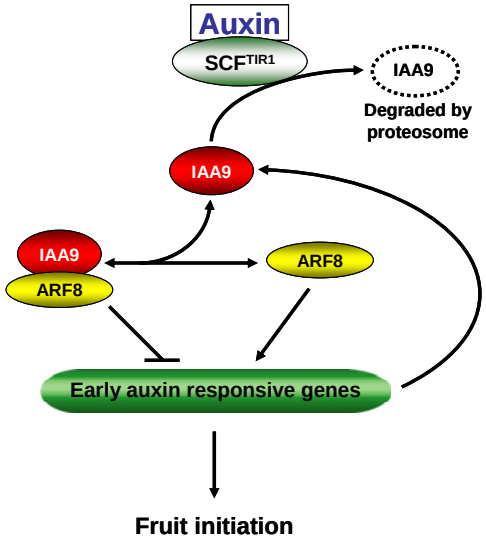

Figure 9. Auxin signaling is mediated by Aux/IAA and ARF proteins.

Specific Aux/IAA proteins (e.g. IAA9) are able to form heterodimers with ARFs as part of complexes that can either directly block transcription of target genes, or act indirectly by preventing the ARF from functioning as a transcriptional activator or repressor. In the example shown here the IAA9 and ARF8 protein complex blocks transcriptional activation and in the absence of IAA9, ARF8 acts as an activator of early auxin responsive genes. Auxin promotes degradation of the IAA9 protein and allows fruit initiation to occur. Similar genes are present in all horticultural crops examined to date, including grapes and citrus.

Based on the results obtained in this project, and the complementary work in tomato (Wang et al. 2005), a model for the role of ARF8 in fruit initiation has been developed in which ARF8/IAA9-like proteins inhibit fruit initiation. Fertilization of the ovule is predicted to cause an increase in auxin levels that prevents the IAA9/ARF8 inhibition and allows fruit initiation to occur. In this model ARF8 is bound to the promoters of a range of primary auxin responsive genes that play an essential role in fruit initiation and development. Transcription of these genes is repressed at this stage by a protein complex, including ARF8 bound to the Arabidopsis ortholog(s) of IAA9, which functions as a repressor. This repressor function of ARF8/IAA9 is likely to occur in ovules (Figure 10). The model further proposes that in WT flowers a fertilization-induced auxin burst promotes degradation of IAA9 which in turn removes the ARF8/IAA9 transcriptional block and allows expression of genes that promote fruit initiation. Whether ARF8 is involved in actively promoting transcription of these genes

is not known, but this could explain why parthenocarpic *arf8* fruit are smaller than fully-seeded WT fruit.

Following removal of the IAA9/ARF8 transcriptional block, an unknown signal is generated that communicates with the rest of the ovary and promotes fruit initiation. While the identity of this signal is not known, possibilities include additional auxin, GA, or cytokinin, all hormones that can promote fruit growth when supplied exogenously to unpollinated flowers. In fact, Approach 2 identified an auxin regulated gene, *SAUR59*, that increases seed-promoted fruit growth and is likely to function downstream of ARF8 to promote fruit initiation and growth. Where SAUR59 exerts this effect is not known, but controlled pollination experiments suggests that it is in maternal tissues such as the ovule integuments or the expanding carpel.

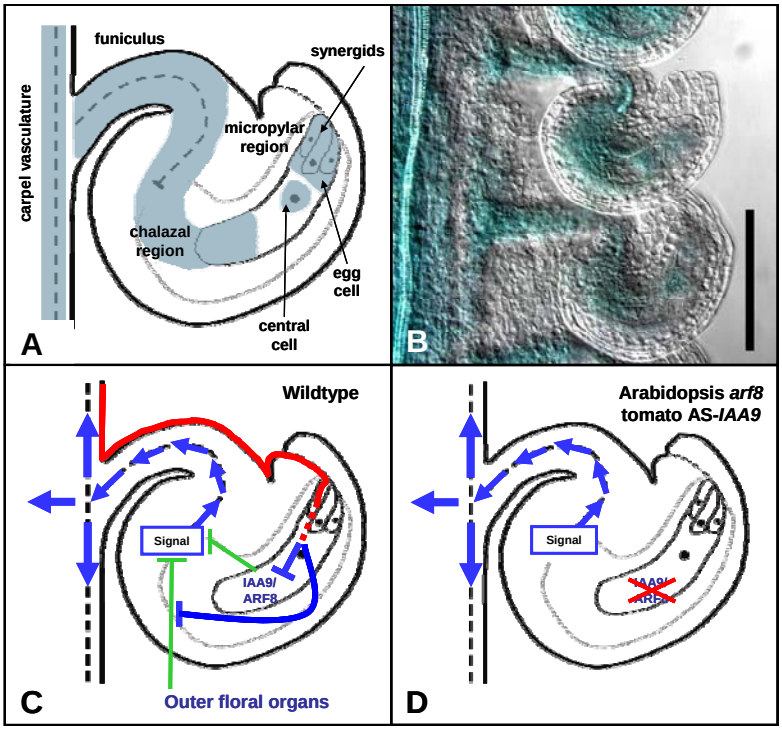

Figure 10. ARF8 expression and model for IAA9/ARF8's role in ovules at fertilization.

A, B. Schematic model of an Arabidopsis ovule and ARF8 expression, based on an ARF8-GUS translational fusion construct, in an ovary at stage 13 (anthesis) (Goetz et al., 2006). The pattern of expression is illustrated by the shading in (A). The embryo sac contains the egg and central cells, each of which is fertilized by a sperm cell released from the pollen tube. **C.** Model for IAA9/ARF8 action in ovules following fertilization. The red line represents a pollen tube. The green lines represent the inhibitory effects of the putative IAA9/ARF8 complex and the unknown signal from surrounding floral whorls. The blue lines represent fertilization-dependent auxin that promotes IAA9 degradation. The blue arrows represent the proposed seed-derived signal that promotes fruit initiation and growth. **D.** In plants either lacking ARF8 with outer floral organs removed (Arabidopsis) or with reduced *IAA9* expression (tomato), fertilization is not required to remove the inhibitory signals and fruit initiation occurs without seed set.

A link between ARF8 and GA is further supported by the partial dependence of *arf8* parthenocarpy on GA activity (Vivian-Smith et al., 2001) and by work with garden peas suggesting that seeds produce a modified auxin which stimulates GA production in the surrounding carpel tissues (Swain and Koltunow, 2006). Approach 2 also demonstrated that combining loss-of-function alleles of 4 of the 5 DELLA-encoding genes (see Figure 2)

enables parthenocarpy and thus provides an additional potential method with which to generate seedless horticultural crops. Importantly, while *arf8*-mediated parthenocarpy is inhibited by an unknown signal from pollen formed within the anther, this does not occur in the della mutant. The results from Approaches 1 and 2 are integrated in Figure 11, which shows the simplest model consistent with the available data.

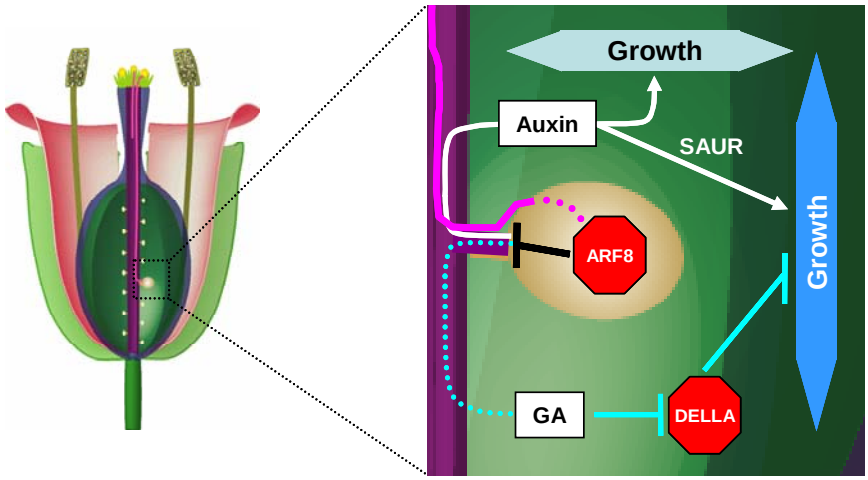

Figure 11. Model for the roles of auxin and GA in fruit growth.

Schematic diagram of a flower at seed fertilization and fruit initiation. The pollen tube (pink) delivers the sperm cells that fertilize the egg (embryo) and central (endosperm) cells. This leads to reduced ARF8 activity and vascular development between the seed and maternal tissues (see Figure 10 for more detail). At this point the seed directly or indirectly stimulates auxin and GA to promote growth of the fruit tissues. Auxin action is likely to involve SAUR genes. Genes such as ARF8 and the DELLAs are excellent candidates for regulating seedless fruit production in horticultural crops.

The proposed roles for auxin and GA in fruit initiation and growth have important implications for a number of major Australian horticultural industries, particularly in regard to existing breeding programs. Applied auxin and GA are commonly used to modify fruit growth in a number of horticultural crops. In proof-of-concept experiments the ARF8 gene has been used to enhance the production of seedless fruit in tomato, while in Arabidopsis both the ARF8 and DELLA genes have been successfully used to generate seedless fruit. In these model plants, as for horticultural crops such as citrus and grapes, application of auxin and/or GA can also lead to seedless fruit production – in other words we have successfully used auxin and GA genes to replace chemical application of these plant hormones. In terms of conventional breeding programs in which seedless fruit production is a key trait, including citrus, table and dried grapes, this knowledge can potentially be used to develop gene based markers. Markers can greatly improve the efficiency of existing breeding programs by allowing selection of the best, i.e. seedless, progeny many years before fruit production occurs. Selection at the young seedling stage can ensure that only seedless progeny are evaluated in the field, increasing the likelihood of the successful development of new elite varieties.

The ARF8 tools developed in the tomato work could be used in tomato breeding to detect expression of the variant and the functional *ARF8* tomato gene and thereby assist in the breeding of better parthenocarpic cultivars in the future. Alternatively, identifying treatments that increase the expression of the variant form of *ARF8* could lead to improved management of tomato lines to induce parthenocarpy under commercial cultivation conditions. However, much additional work needs to be done to determine which growth conditions lead to the generation of the variant and whether particular conditions can increase the levels of the

variant to increase parthenocarpic fruit set. This research would need to be linked to a viable breeding program to generate outcomes as sufficient know-how has not yet been generated. In the absence of this research, there is no obvious protectable IP position and CSIRO will not be continuing work on tomato in the absence of both funds and a current Australian parthenocarpy breeding program. This work has therefore been prepared for publication.

Colour, mouth feel and health properties

The study of anthocyanin and tannin synthesis in Arabidopsis and fruit species has led to a number of excellent publications and clearly shows the synergistic effect of having three concurrent projects in similar areas, including the CRCV project to study the formation of grape flavonoids.

Approach 3 focused on flavonoids such as anthocyanin, which are involved in fruit colour and antioxidant levels, and tannins, which contribute to flavour and mouth feel. This research identified genes that regulate the anthocyanin/tannin synthesis pathway in the model plant Arabidopsis as prerequisite for work in target crops. Initially the step performed by the leuco anthocyanidin dioxygenase (LDOX) enzyme was elucidated by the isolation and of this gene through a mutant based approach in Arabidopsis (Figure 12; Abrahams et al., 2003). This result confirmed that cyanidin was the preferred substrate for tannin synthesis in Arabidopsis. For understanding the regulation of the pathway the PAP genes were studied in detail and each was found to be able to regulate anthocyanin biosynthesis in the plant, though PAP3 is the weakest of the regulators. They each have distinct expression patterns suggesting that they work in a coordinated fashion to give the plant maximum benefit and flexibility in the synthesis of anthocyanins.

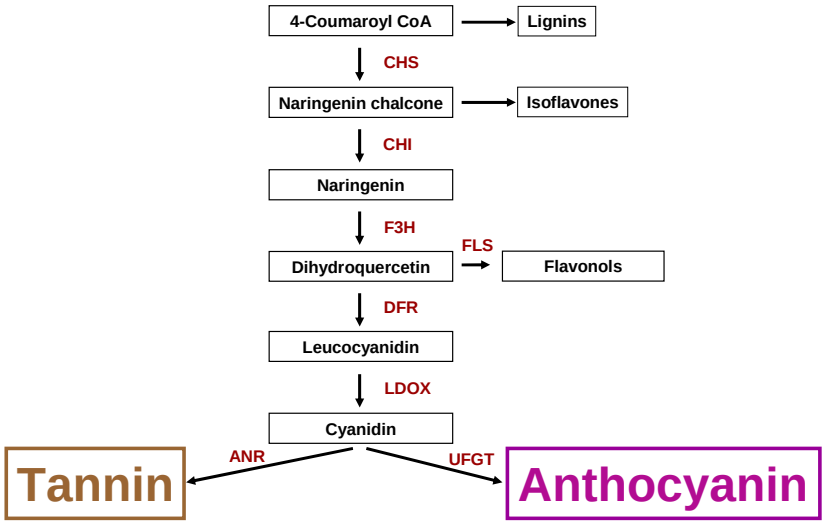

Figure 12. Pathway of tannin and anthocyanin production in Arabidopsis.

Plants are able to separately regulate the production of either tannins or anthocyanins using the PAP genes (anthocyanin) and related genes such as TT2 (tannins). Identifying the domains and protein motifs that differ between the four PAPs and TT2 would enhance our ability to manipulate the two branches on the pathway in target crops. This information would also simplify the initial identification and characterisation of anthocyanin/tannin regulators in target crops. The work has successfully isolated a previously unidentified protein region that appears to be important in controlling specificity.

Approach 3a has been very successful in elucidating the genes encoding structural enzymes and the light-regulation of the anthocyanin and tannin pathways, in addition to the isolation and characterisation of the anthocyanin regulator in apples. This component of the research has:

- Determined the flavonoid composition of apples throughout fruit development and ripening
- Identified apple structural genes for anthocyanin, flavonol and tannin synthesis
- Determined the pattern of expression of structural genes during fruit development
- Determined the influence of light exposure on apple flavonoid composition and gene expression
- Discovered that all flavonoid pathway genes are co-ordinately regulated during fruit development and by light – indicates the pathway is controlled by regulatory genes
- Identified several candidate regulator genes that may control flavonoid synthesis in apples
- Identified and characterised MdMYB1 as a key regulator of colour
- Determined that the expression of *MdMYB1* was much lower in green skin compared with red skin – shows that alleles of *MdMYB1* might be different in red and green apples
- Produced a marker for skin colour (red/green) based on a difference in the DNA sequence of the promoter of the *MdMYB1* gene
- Validated the use of the marker for predicting red or green skin colour in progeny of a ST24/49 X Golden Delicious cross

The most significant finding of this research project is the identification of the *MdMYB1* regulatory gene which appears to be the key regulator of anthocyanin synthesis in the skin of apples during ripening. This gene shows much lower expression in green skin than red skin and is responsive to sunlight in parallel with colour formation. Identification of this key gene has already led to the development of a DNA marker for skin colour in selected germplasm and further validation of this result would lead to a robust DNA marker for skin colour (Figure 13).

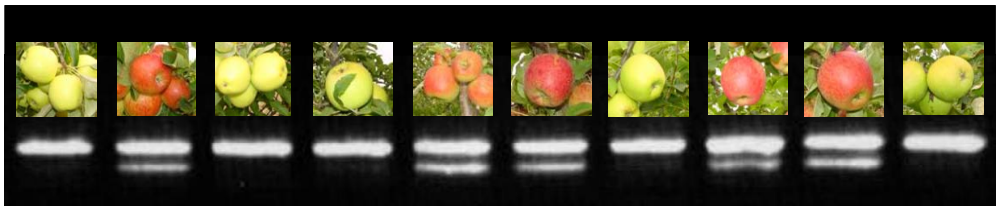

Figure 13. The Aushort Key Genes project has identified a gene-based marker for apple skin colour. The result of the marker test for different individuals is shown by the white bands under the photos of fruit from each plant tested. Two white bands indicate the presence of the gene for red skin colour. This test could be used to predict eventual fruit colour in young seedlings several years before the trees set fruit in a breeding orchard.

Because this marker was designed to utilise the difference between the red and green alleles in Lady Williams and Golden Delicious, it should correlate consistently with skin colour in progeny containing germplasm from these parents. In a population of several thousand individuals, it would be very unlikely that the marker would not predict colour correctly. From a practical viewpoint, the new marker for skin colour is easy to use and gives definitive results. However the marker has been tested for only a few apple cultivars and is known not to predict colour correctly for Granny Smith and the related Grandspur. For use in an extensive breeding program with diverse germplasm, the marker would need to be tested for utility with the relevant parental cultivars, while a different marker would be required for use with Granny Smith germplasm. The marker cannot be used to predict colour intensity or patterning.

The apple work also provides the potential for new avenues of research, which could lead to major breakthroughs with implications for the breeding program. For example, in the promoter region of the gene, there are several differences between red and green alleles, one of which has been identified as a potential switch that responds to light. Similar sequences in other species have been shown to be part of the light regulation of gene expression. This discovery provides the opportunity to investigate how light switches on the *MdMYB1* gene resulting in anthocyanin synthesis to determine if there is the potential to develop tools for breeding fruit that do not rely on direct sunlight for even and consistent skin colouration.

Discussions are ongoing between CSIRO and the major players DAFWA, APAL, HortResearch NZ and HAL with regard to the future of apple research at CSIRO.

Conclusion

The Aushort Key Genes for Horticultural Markets project has met and exceeded all of its original goals. This project has used model plants and advanced molecular-genetic techniques to discover and characterise novel genes that can be used for the genetic improvement of key production and consumer traits in a wide range of horticultural crops. In apple a gene that confers red skin colour has been identified and used to generate a marker that could be used in existing breeding programs. The fact that this project will not be continued means that, without additional investment from industry, this research will cease. This will significantly limit the ability of Australia's horticultural industries to benefit from biotechnology and ultimately to maintain and improve Australia's ability to successfully compete in world markets.

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Mandy Walker 30 November 2006, Press release: "Found – the apple gene for red"

Media coverage: The apple gene for red

Date	Topic or Headline	Publication	Category
30-Nov-06	Colour control in apples	ABC Radio Darwin	Radio
30-Nov-06	Colour control in apples	ABC Radio Mid West WA	Radio
30-Nov-06	Found - the apple gene for red	EurekAlert (www.eurekalert.org)	Internet
30-Nov-06	Gene for red apple colour discovered	AP Foodtechnology.com	Internet
30-Nov-06	Gene for red apple colour discovered	Nutra Ingredients - USA	Internet
30-Nov-06	Scientists crack colour code for blue apples	News.Com	Internet
01-Dec-06	Apples get genetic spit and polish as scientists seek perfection	Sydney Morning Herald (M-F) Newspaper	Newspaper
01-Dec-06	Apples' new polish	Sydney Morning Herald (M-F)	Newspaper
01-Dec-06	Aust scientists claim they beat NZers to punch on apple colour	NZPA Newswire	Newspaper
01-Dec-06	Australia: scientists crack colour code for blue apples	Fresh Plaza (www.freshplaza.com)	Internet
01-Dec-06	Bossy genes add up to some nice fruit	Age (Monday - Friday)	Newspaper
01-Dec-06	Found - the apple gene for red	Biology News (www.biologynews.net)	Internet
01-Dec-06	Found - the apple gene for red	PhysOrg.com	Internet
01-Dec-06	Gene to control apple colour	NSW - Metropolitan Radio	Radio
01-Dec-06	Research red letter day for apple lovers	Adelaide Advertiser - Mon-Fri	Newspaper
01-Dec-06	Scientists work on apple hues	Manawatu Standard (NZ)	Newspaper
02-Dec-06	Apple gene for red found by Australian scientists	China View (http://news.xianhuanet.com)	Internet
02-Dec-06	Aussies claim apple research	Hawkes Bay Today	Newspaper
03-Dec-06	Gene behind red color of apples identified	All Headline News (www.allheadlinesnews.com)	Internet
04-Dec-06	Found - the apple gene for red	CSIRO Monday Mail	Internet
04-Dec-06	Gene to control apple colour found	ABC Radio North West WA	Radio
04-Dec-06	Gene to control apple colour found	ACT Metropolitan Radio	Radio
04-Dec-06	Gene to control apple colour found	SA - Metropolitan Radio	Radio
04-Dec-06	Like red apples? Gene research finds secret	farmonline	Internet
04-Dec-06	Why an apple turns red	Scenta (www.scenta.co.uk)	Internet

05-Dec-06	Apple gene for red	Port Lincoln Times	Newspaper
05-Dec-06	Aussies claim they beat Kiwis to punch on apple colour	Ashburton Guardian (NZ)	Newspaper
05-Dec-06	Australians claim genetic breakthrough	Nelson Mail (NZ)	Newspaper
05-Dec-06	Discovery of 'red gene' points to bad apples	Live Science (www.livescience.com)	Internet
05-Dec-06	Gene to control apple colour found	ABC Radio South Coast (WA)	Radio
06-Dec-06	Apple red in genes	Northern Guardian	Newspaper
06-Dec-06	Gene to control apple colour found	ABC Radio North West WA	Radio
06-Dec-06	Gene to control apple colour found	ABC Radio Riverina	Radio
07-Dec-06	Apples give up secrets	Tasmanian Country	Magazine
07-Dec-06	On the road	North Queensland Register	Newspaper
07-Dec-06	Rainbow apples	North Queensland Register	Newspaper
13-Dec-06	Gene that makes apples red	ABC Radio Riverland SA	Radio
14-Dec-06	Brighter and redder apples	ABC Radio North and West SA	Radio
18-Dec-06	How many colours can an apple be?	Canberra Times (M-F)	Newspaper
27-Dec-06	Apples to get a colour lift	Weekly Times	Newspaper
01-Jan-07	Apple gene find	Southern Farmer	Magazine
15-Feb-07	Found - red apple gene	Good Fruit and Vegetables	Magazine
15-Feb-07	Red apples	Food and Drink Business	Magazine
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RECOMMENDATIONS

- CSIRO and HAL, together with appropriate industry representatives, should develop a long-term strategy for effective investment in, and use of, biotechnology in Australian horticulture to maintain Australia's competitive position in world markets.

- Ensure that Australian horticulture benefits from the research in the Key Genes project by using the new knowledge and tools to improve the efficiency in delivering improved new varieties through major Australian breeding programs.

- The citrus, table and dried grape industries are best positioned to benefit from the outputs relating to seedless fruit production and should explore mechanisms to develop gene-based markers that can be used to improve breeding efficiency.

- The pome fruit industries, particularly apple, should endeavour to build on the progress so far in developing a marker for apple skin colour and develop additional markers to follow more sophisticated colour, health and flavour traits in breeding programs.

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