Novel genetic marker technologies revolutionize apple breeding©

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INTRODUCTION

For the past 24 years, my vision and research focus have been to lead the development of modern tools for fruit breeding at Plant & Food Research (PFR). My initial focus was very specific, on apple, however this has expanded, so that our "Mapping & Markers" team of genetic mapping researchers now works on a range of crops, including apple, pear, kiwifruit, peach, apricot, raspberry, blueberry, blackcurrant, hops, and most recently, manuka, applying the same DNA-based technologies to all. We work closely with industry and breeders for each crop, as well as many other PFR and international scientists, including genomics scientists focussing on DNA, those who work on pests and diseases, or fruit characters, such as flavour and texture, using our genetics and genomics expertize to develop tools to "breed better cultivars faster" (van Nocker and Gardiner, 2014) for international consumers and New Zealand growers. I will discuss some of the novel features of the modern apple breeding programme at PFR, which has led the world in applying new fruit breeding technologies. This programme employs marker assisted selection for critical "must have" characters in large seedling populations, which can include thousands of seedlings, in order to increase the efficiency of developing new apple cultivars with the features sought by consumers. I will discuss in some detail a specific example of how marker assisted selection is being applied by our breeders to speed up the development of rootstock varieties with the very specific characters needed in the rootstocks of tomorrow.

THE PFR BREEDING PIPELINE TODAY

The pipeline currently used by our apple breeders is illustrated in Figure 1. As has always been the case, the primary raw material for breeders is the treasure house of existing cultivars and other germplasm in the research orchard. Another, more recently developed resource comprises DNA-based information about apple. In 2010, the sequence of the apple genome was published (Velasco et al., 2010) and this includes around 57,000 sequences of genes, that together determine what goes to make up the apple tree and fruit, and control their development. From the point of view of the scientists in my "Mapping & Markers" team, these genes are all potential candidates for developing "genetic markers" that are associated with the control of a specific trait and we use to select elite seedlings that carry a particular combination of traits from large breeding populations. Our task is to work with genomics scientists to narrow down the candidates to one for each trait of value to breeders and then develop a marker that maps to the trait, at a defined position on one of the 17 apple chromosomes (Gardiner et al., 2007). The choice of the specific traits we work on depends on industry needs and priorities. In general, these are traits that characterize a crisp, juicy, flavoursome, and attractive apple at the point of sale to the consumer. New cultivars will require a minimal input of sprays by the grower to control pests and diseases, as natural genetic resistances against common pests such as apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*), woolly apple aphid (*Eriosoma lanigerum*), and fire blight (*Erwinia amylovora*), will be bred into both scion and rootstock cultivars. The trees of new scion cultivars will be dwarfed, fruiting earlier after planting, with a high yield per hectare because they are grafted onto rootstock cultivars that confer these grower-friendly traits to the scion.

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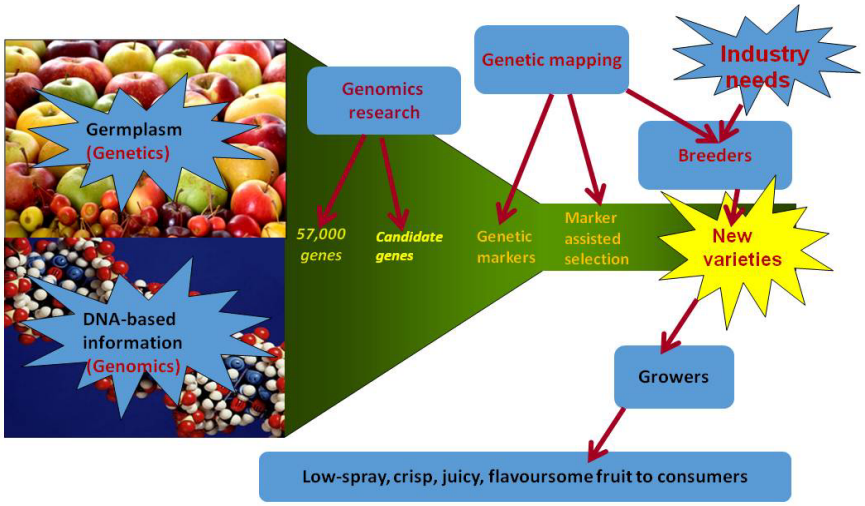

Figure 1. Apple breeding pipeline. The pipeline is driven from top right (industry needs). The process is depicted from left (resources of germplasm and DNA-based information) through far right (new varieties) and to bottom (fruit to consumers). Inputs from genomics research, genetic mapping and breeders occur sequentially, following definition of industry needs.

The role of the apple breeder is to combine these desired attributes by making new crosses and to select the best seedlings to proceed to new cultivars, or as parents for further crosses. To select parents to make crosses, he or she needs to use knowledge of the germplasm available, both existing old and new cultivars, as well as previous selections that carry the traits of interest. My team's role is to assist the breeder in selecting the parents for new crosses and subsequently the genetically elite seedlings that carry the "best" combination of traits in the resulting progeny populations. To achieve this, we work in partnership with the breeders and use genomics information to develop genetic markers that are associated with a particular trait and can hence be used to select plants that have the genetic potential to express the associated character. These genetic markers can be compared with DNA fingerprints and are derived in the best case from a candidate gene that controls the expression of the trait, e.g., for resistance to apple scab, fire blight or powdery mildew, and/or red fleshed-fruit; or are located on one of the 17 apple chromosomes, so close to the controlling gene that they are generally inherited together. Examples here could be resistance to woolly apple aphid, or the dwarfing of a grafted scion by a rootstock.

MARKER ASSISTED SELECTION

Marker assisted selection is the process of using these genetic markers as diagnostic tools, to identify plants that carry the desired traits, by screening DNA extracted from each plant for the presence or absence of the form of the marker associated with the trait. These plants may be potential parents, or seedlings derived from crosses made by the breeders. Newly germinated seedlings can be screened as soon as they have a few leaves, because the amount of tissue required for DNA extraction is only the size of a small fingernail. The most effective use of marker assisted selection in seedling populations is to screen for a combination of "must have" traits. In scion cultivar breeding, these include resistance to apple scab, powdery mildew and woolly apple aphid (Gardiner et al., 2007), red skin (Chagné et al., 2016) and flesh colour (Chagné et al., 2013), while some breeders also use markers for genes that contribute to complex fruit traits, such as texture (Longhi et al., 2013). In rootstock breeding, "must have" traits are also pest and disease resistance, as well as the absolutely critical trait of the capability to dwarf a scion grafted onto the rootstock.

In the case where a parent that is heterozygous for any specific trait (carries one copy of the gene) is crossed with a susceptible parent and the trait is controlled by a single gene, only half the seedlings in the population from the cross will carry that trait, according to

Mendelian inheritance. Seedlings that do not carry the marker for this trait can be discarded, as these are not of further interest to the breeder. Only the seedlings carrying the "must have" trait need be screened for the next trait, after which the number of the seedlings of interest can again be potentially halved. The relatively few genetically elite seedlings that remain after the screening of seedlings for all "must have" traits that the breeder has included in a specific cross are planted in the research orchard. Here, the breeders examine them closely for the level of expression of these traits, as well as for other traits that have not been screened for using markers.

Because the size of the population has been reduced following the marker screen, breeders can afford to accelerate the development of the smaller number of elite seedlings, using environmental treatments that promote flowering and so reduce the time between generations. Culling of unwanted seedlings before planting in the field reduces orchard costs for each population, as well as expensive and laborious screening of very large numbers of seedlings for some traits, for example ability of fruit to store well, or the dwarfing trait in rootstocks. An important feature of marker assisted selection is that the seedlings need not be mature enough to express the trait of interest. This is most significant for the selection of traits expressed in the mature tree only, i.e., any fruit trait, such as flesh colour and crisp texture. A novel application of marker assisted selection is to identify plants that carry more than one resistance to a particular pest or disease. Such plants are termed to carry "pyramided resistances" and have the advantage of reducing the possibility of the pest or disease overcoming the resistance, as it is simpler for a pathogen to overcome a resistance controlled by a single gene, than by two different genes. To develop such plants, the breeder crosses parents carrying resistances from different sources, then uses genetic markers specific for the different resistances to select only the seedlings carrying both (Bus et al., 2009). These will be 25% of the initial population for resistances each controlled by an independently segregating single gene and inherited in a Mendelian fashion.

Marker assisted selection of genetically elite seedlings has been employed in the PFR apple breeding programme as a tool to breed better cultivars faster for more than 10 years, with more than a combined 25,000 seedlings now screened annually in the scion and rootstock cultivar breeding programmes.

EXAMPLE OF MARKER ASSISTED SELECTION IN THE APPLE ROOTSTOCK BREEDING PROGRAMME

Commercial apple production depends on the grafting of scion cultivars onto rootstocks as the method of propagation. The choice of rootstock is important, as not only is the stature of the scion markedly reduced by grafting onto a dwarfing rootstock compared with a vigorous rootstock (Figure 2), hence making management and harvesting of the trees much easier, but there is a positive effect of using dwarfing rootstocks on orchard production traits, including a reduction in time to fruiting following grafting and a yield increase per hectare.

Four years ago, our rootstock breeder Vincent Bus came to us with two populations that had been developed to incorporate new traits into future dwarfing rootstock cultivars. Crosses had been carefully designed, whereby both populations would carry the dwarfing trait controlled by *Dw1*, inherited from both parents (Figure 3), so that the seedlings would include some that carried two copies of *Dw1*. In addition, each population carried the *FB_R5* resistance to fire blight, mapped to chromosome 3 of *Malus* × *robusta* 5 (Peil et al., 2009; Gardiner et al., 2012) as well as resistance to woolly apple aphid conferred by the genetic locus *Er2* on apple chromosome 17 (Bus et al., 2008) of *Malus* × *robusta* 5. The latter was inherited from one parent in population one and both parents in population two. Population one additionally carried the different *Er3* woolly apple aphid resistance from chromosome 8, originally from *M. sieboldii* 'Aotea 1' (Bus et al., 2008), inherited from one of its parents. Leaves had already been harvested from the seedlings for DNA extraction and were stored freeze-dried in our laboratory. Vincent wished to identify the dwarfing seedlings that also carried fire blight and woolly apple aphid resistance from one or both sources. Both the DNA extraction from the stored leaves and marker screens were performed efficiently in less than

2 weeks by Slipstream Automation Ltd (http://www.slipstream-automation.co.nz). This service company was established by a former team member and specializes in performing automated DNA extraction and subsequent genetic marker screens, including cherry-picking out marker positive DNA samples to carry forward to the next screen, hence reducing screening costs per plant.

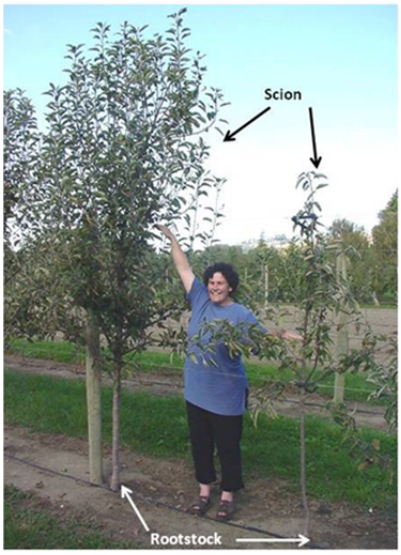

Figure 2. Comparison of relative size of scions grafted on vigorous (left) and dwarfing (right) rootstocks, two years after grafting of scion to rootstock.

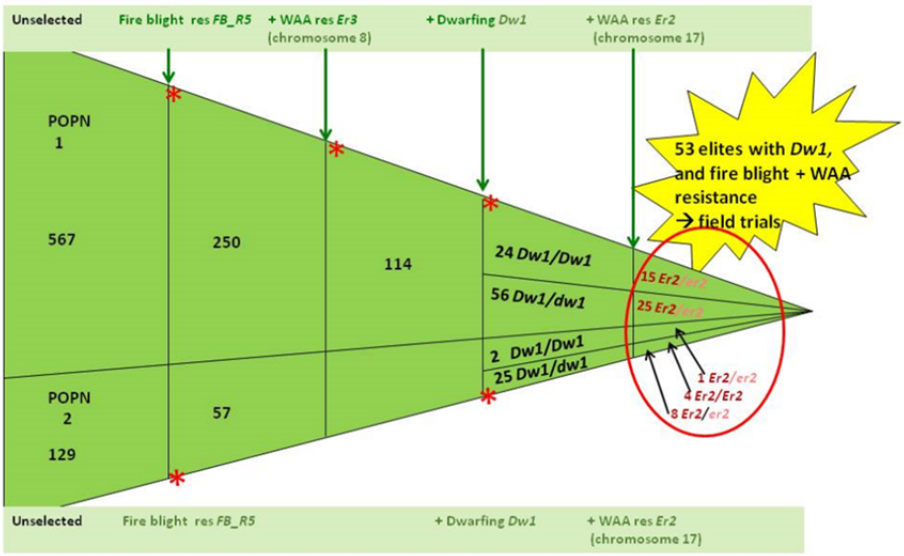

Figure 3. Protocol for efficient rootstock marker assisted selection. Two breeding populations of 567 and 129 seedlings respectively were screened sequentially with markers for *Fb_R5* (fire blight resistance), *Er3* (woolly apple aphid resistance on chromosome 8) screened over population 1 only, *Dw1* (dwarfing of grafted scion) and *Er2* (woolly apple aphid resistance on chromosome 17). Green arrows indicate timing of each marker screen. Red asterisks * indicate timing of selection of marker positive seedling DNA for the subsequent screen. Numbers of seedlings at each of the 5 stages (unselected through to fully selected population) are indicated.

Figure 3 shows the protocol for the genetic marker screening over the total of 696 individual DNA samples. Marker screens were performed in a specific order, with the cheapest marker first (*Fb_R5*), followed by the next cheapest (*Er3*) and finally the most costly marker screens, which were for the genetic loci *Dw1* and *Er2*, with no sample picking employed between these. The first screen with *Fb_R5* approximately halved the number of seedlings to be screened with the next marker from 696 to 307, as predicted by Mendelian inheritance. Population one was more than halved again following screening with the *Er3* marker. Next came the very important screen over both populations for the genetic locus *Dw1*, where the specialized marker employed was able to identify not only the 107 seedlings that carried the locus, but also the number of copies (alleles) carried by each seedling, as it can distinguish between the heterozygous and homozygous states. The final screen for *Er2* revealed 53 genetically elite seedlings that carried the dwarfing trait, as well as resistance to fire blight and woolly apple aphid. Vincent chose all of these seedlings to advance to orchard trialling to validate the expression of the traits selected for, as well as other traits for which markers were not yet available. Forty of these seedlings exhibited the *Er2* resistance pyramided with *Er3*, thus enhancing the potential durability of resistance to woolly apple aphid. Seedlings that were identified as homozygous for either *Dw1* or *Er2* would theoretically make ideal breeding parents, as all progeny would carry the genetic locus. The effect of the homozygosity on the expression of these traits will be investigated during the course of the orchard trial.

The 53 elites selected for the field trial represented 7.6% of the initial seedling population. Such a large reduction in population size has enabled a restructuring of the orchard trial protocol, as shown in Figure 4. The starting material for both the conventional protocol in the absence of marker assisted selection and the new protocol is seedlings that have been pre-selected for lack of obvious adverse traits after growing in the nursery for 1 year. These unwanted characters include spines and burr knots. The conventional protocol involves initial grafting of each seedling with a scion cultivar, then evaluating each plant over 3-4 years for orchard production traits exhibited in the scion, such as extent of dwarfing, time to first flowering and fruit yield. About 10% of the rootstocks are selected at this stage to go forward to evaluation for stool-bed production traits. Plants that root easily and demonstrate good stool-bed production traits, e.g., many stools of the right size, are selected after 5 years of monitoring in the stool-bed, to proceed in replicate to orchard production trials following grafting with scions. This process takes another 5 years before selections are made of the best performing rootstocks for potential advance to new cultivar status. The complete protocol takes about 14-15 years starting from seed. In the new protocol where marker assisted selection is employed, the number of seedlings entering the orchard trial is reduced by over 90%. The initial orchard production trial performed on single trees in the conventional protocol is bypassed and rootstocks proceed immediately to the stool-bed production trait trial. The plants are closely monitored, so that ones that root most easily are speedily identified, grafted with scions and advanced to the replicated orchard production trait trials after 3-4 years. The important production trait trials last 5 years, as in the conventional protocol; however, the new protocol incorporating marker assisted selection reduces the time from seed to potential cultivar by 5 years, compared with the previous protocol. In addition, because fewer seedlings are assessed in the orchard trial, costs for both land and for labour involved in trait assessment are reduced. This is a revolution!

The protocols for marker assisted selection and field trials in the apple rootstock breeding programme are constantly developing as new traits are introduced into the crosses that Vincent makes annually and as our team develops genetic markers for these traits. We can now screen for a second genetic locus influencing dwarfing (*Dw2*) that enhances the effect of *Dw1*. This will be important in the development of rootstocks with a greater or lesser dwarfing effect on grafted scions. In the past year, the seedlings carried a genetic locus which has been shown to control root formation and we were able to use a published genetic marker (Moriya et al., 2015) to identify seedlings carrying this trait, which still will need to be validated. Screening for a third genetic locus for woolly apple aphid resistance (*Er1*), mapped to chromosome 8 (Bus et al., 2008), was introduced to increase the options for

resistance pyramiding.

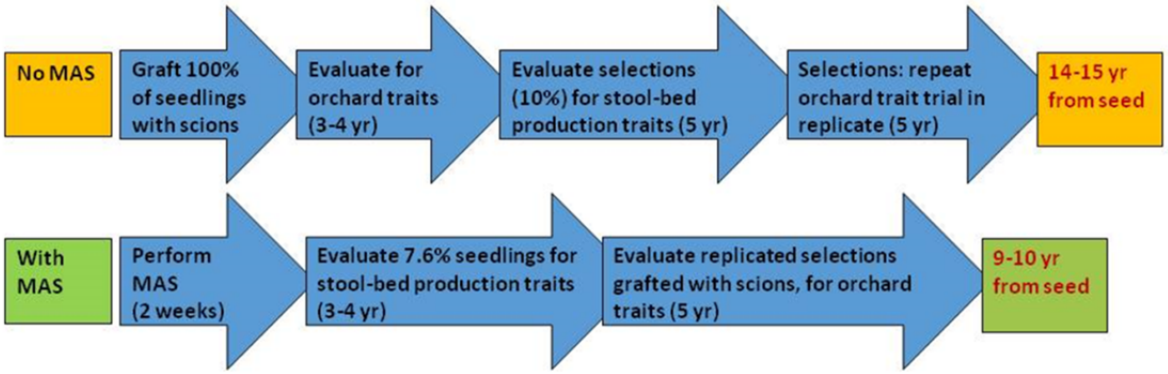

Figure 4. Comparison of orchard trial protocols for examining performance of apple rootstocks, with and without the application of marker assisted selection.

FUTURE TRENDS

Our team is steadily developing new genetic markers for traits that are important to consumers and consequently our apple breeders. These include resistances to additional pests and diseases, such as apple canker, as well as traits associated with fruit quality, particularly texture. We are working to transform existing genetic markers into improved forms that are highly reproducible across different seedling populations from different genetic origins, as well as being easier to use and more amenable to automated screening. We are working with other PFR scientists to introduce the new technology of genome wide selection into our apple breeding (Kumar et al., 2012). This process considers thousands of genetic markers across the 17 chromosomes of apple at the same time and enables the selection for or against complex characters controlled by a number of genetic loci, each with small effects, but which when taken together are significant.

In summary, new genetic marker technology has indeed revolutionized apple breeding. The revolution is not yet over and the future will be exciting.

ACKNOWLEDGEMENTS

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