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MINI REVIEW

Breeding better cultivars, faster: applications of new technologies for the rapid deployment of superior horticultural tree crops

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Woody perennial plants, including trees that produce fruits and nuts of horticultural value, typically have long breeding cycles, and development and introduction of improved cultivars by plant breeders may require many breeding cycles and dozens of years. However, recent advances in biotechnologies and genomics have the potential to accelerate cultivar development greatly in all crops. This mini-review summarizes approaches to reduce the number and the duration of breeding cycles for horticultural tree crops, and outlines the challenges that remain to implement these into efficient breeding pipelines.

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INTRODUCTION

A survey of the produce section of a typical supermarket will reveal a stunning diversity of shapes, sizes and colors of myriads of vegetables, fruits and nuts. Almost all of today's commercial produce reflects the results of continuing breeding programs, most focused on longstanding goals such as quality, storage potential, yield, color and size. However, although such efforts have been successful in generating commercial cultivars that bear high quality produce under the best current production regimes, breeders generally lack the capacity to generate new cultivars quickly in response to evolving consumer preferences and crisis situations.

This limitation is especially evident for tree fruits and nuts, products of breeding cycles that can extend to a dozen years or more. Like other sectors of horticulture, the fruit and nut industries face highly dynamic situations arising from such factors as decreasing labor availability, increasing environmental concerns, cost of energy, climate change and epidemics of new and invasive insects and diseases. In addition, the inability to re-program tree form and phenology quickly limits deployment of highly efficient production technologies. The generally reactive, rather than proactive, nature of response to these factors translates to the release of new cultivars only after such pressures have accumulated significant impact on production.

An excellent example is the devastating citrus greening (Huanglongbing), a bacterial disease of citrus crops vectored by sap-sucking psyllid insects. The disease has a broad target range, including the economically important grapefruit (*Citrus* × *paradisi*), orange (*C.* × *sinensis*) and tangerine (*C. tangerina*).[1] There is no effective management strategy for this disease once trees are infected, and trees must be destroyed to prevent further spread.[2] Citrus greening has affected production worldwide, and although detected in Florida only in 2005, has already cost industries in that state well over US$4 billion.[3] Although natural resistance might exist, for example in some genotypes of the related pomelo (*C. maxima* syn. *C. grandis*),[4,5] such a simple genetic patch for elite cultivars necessitates a series of backcrosses to recover the original traits of the elite cultivar. Since the breeding cycle in citrus ranges from 5 to 10 years, this may preclude any rescue of the Florida industries through rapid introduction of resistant cultivars.

For citrus and other woody perennial tree fruit crops, the main factor driving the term of the breeding cycle is the length of the juvenile phase. Juvenility is defined as the extended period of post-germination, vegetative development in which flowering is repressed even under otherwise favorable environmental conditions.[6] The length of the juvenile phase for tree fruit crops has been variously reported to extend from at least three years (peach) to 15 or more years (avocado) (Table 1). In nature, juvenility ensures that flowering is not initiated before the plant has the photosynthetic capacity to produce fruit and viable seed, or that resources are not diverted to flowering before a plant has reached a competitive size in its environment.[16] However, this natural safeguard can be subverted by breeders, who are able to maintain plants under optimal growth conditions, and often need only to obtain pollen to advance to the next generation.

To be fully effective for rapid cultivar development, shortening the breeding cycle must be linked to a reduction in the number of breeding cycles. For tree fruits and nuts, the past 10 years have witnessed the initiation of a transition from traditional breeding techniques, based largely on phenotype, to genome-assisted breeding approaches, based on previously cataloged trait-locus associations, resulting in fewer breeding cycles. In addition, efficient genotype selection is most advantageous where a reduction in the length of breeding cycle is possible. This interdependence is leading to a groundswell of interest in promoting rapid-cycle breeding as an integrative study, as evidenced by the recent *International Rapid Cycle Crop Breeding Conference*, held in January 2014 in the Washington, DC area.[17–23]

REDUCING THE LENGTH OF THE BREEDING CYCLE: MANIPULATING CULTURAL CONDITIONS

In woody perennial plants, the length of the juvenile period is influenced by environment[16] and is inversely correlated with vigor.[24]

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Table 1. Length of the juvenile phase under field conditions for selected tree fruit/nut crops

Crop	Duration (years)	Source
Almond	3–4	[7]
Cherry	3–5	[8]
Peach	⩾3	[9]
Pistachio	4–10	[10]
Walnut	5–9	[11]
Orange	5–10	[12]
Lemon	5–10	[12]
Mandarin	5–10	[12]
Tangerine	5–10	[12]
Pecan	⩾5	[13]
Apple and Pear	6–12	[14]
Avocado	15+	[15]

Accordingly, environmental conditions that reduce vigorous growth, such as mineral deficiency, low light, water stress, defoliation or cold stress, tend to delay the transition from the juvenile to adult phase, whereas the conditions that allow for vigorous growth can shorten the period of juvenility.[25,26] In apple (*Malus* × *domestica*), where field-grown seedlings typically do not flower until they are at least 5 years old, plants can be promoted to the adult reproductive phase after as little as 10 months under optimal growth conditions.[26] The apical portion of the plant, having attained the adult state, can be grafted to a rootstock for further growth and maintenance (Figure 1). A drawback of such an approach is that plants can grow very tall and become difficult to manage in a controlled environment or greenhouse setting.[27]

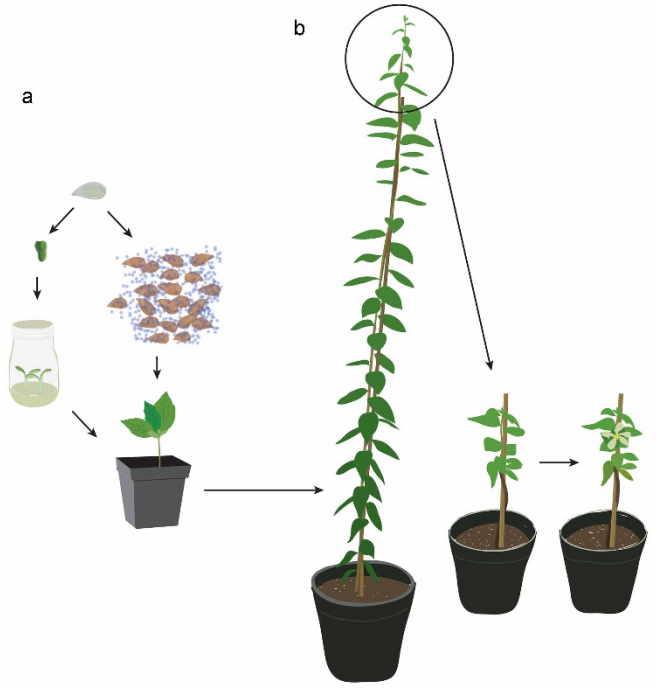

Figure 1. Approaches for acceleration of the breeding cycle through manipulation of cultural conditions. (**a**) Embryo rescue or chemical treatment of the seed to break seed dormancy offers a shortcut to flowering. (**b**) Maintaining seedlings under optimal growth conditions can greatly abbreviate the juvenile phase. Typically, plants may become very tall, and the apex can be grafted to a rootstock for further growth and maintenance. Chilling may be required for effective floral development.

Growth can often be managed with plant growth regulators (PGRs), once any potential adverse effects on accelerating the phase transition have been assessed. The activity of PGRs, including phytohormones, on flowering in seedlings of woody perennials has been fertile ground for research, and many striking effects have been reported.[27] However, effectiveness and consistency of PGR treatments have generally shown high variability among genotypes, species and experiments.[27] In addition, such studies are often obfuscated by lack of distinction between effects on phase change and effects on flowering. Accordingly, the use of PGRs as a method for shortening juvenility and/or promoting flowering has not been widely adopted as a general tool for reducing the length of the breeding cycle; however, it is being applied to reduce extension growth where space is at a premium (R Volz, pers. commun.).

Woody plants 'forced' to the adult phase by optimal growth may still need to undergo a period of up to 10 weeks of chilling and/or defoliation for effective floral development[26] (Figure 1). Interestingly, transgenic plants ectopically expressing the flowering gene *FT* largely escape the chilling requirement for normal floral development (see below), suggesting that chilling influences events relatively early in flowering pathways. The endogenous genetic mechanism of chill requirement is a fascinating area for further research and potentially an additional target for reducing the length of the breeding cycle.

Overcoming seed dormancy is another potential shortcut. Most flowering plants produce seed that is initially dormant, requiring time and/or specialized environmental conditions including specific moisture, temperature, and light quantity and quality for germination. In nature, this phenomenon minimizes the possibility of germination until conditions optimal for seedling growth are encountered. For most temperate-zone tree fruits and nuts, dormancy is naturally overcome by extended periods of low winter temperatures under high moisture conditions. *In vitro*, up to 12 weeks of low-temperature stratification may be required to break dormancy. For rapid-breeding protocols under controlled-environment conditions, this can add significantly to the length of the breeding cycle. Consequently, approaches to bypass seed dormancy are increasingly employed (Figure 1). One of these consists of dissecting the embryo from the seed at a stage of development before dormancy is imposed, and culturing the embryo under conditions that promote direct seedling development. This technique is also used in rescue of embryos, typically derived from wide crosses, which otherwise may not produce viable seedlings.[28,29] Another approach, which is more compatible with large numbers of seeds, is to promote germination through treatment with phytohormones. Because germination is generally mediated by the balance of (repressive) abscisic acid (ABA) and (promotive) gibberellins (GAs) within the seed, dormancy can often be largely bypassed by application of bioactive GAs. Nitric oxide is increasingly known as a strong dormancy-releasing agent in many species[30] and shows new potential for promotion of germination in horticultural crops, especially where GA effects might be detrimental to seedling growth, or might repress early flowering.

PROMOTING FLOWERING THROUGH BIOTECHNOLOGY

Possibly the most exciting potential for reducing the length of the breeding cycle is the biotechnological manipulation of endogenous, genetic flowering pathways. Nearly 20 years ago, Detlef Weigel and Ove Nilsson showed that flowering could be triggered in aspen by transgenic expression of a gene from *Arabidopsis* called *LEAFY (LFY)*.[31] In the past two decades, there has been appreciable refinement of this pioneering technology, including the employment of additional flowering genes, use of inducible promoters to drive transgene expression, and recently, approaches to transmit the transgenic stimulus through grafting.

To appreciate this potential, it is necessary to understand those genetic mechanisms of flowering that seem to have been widely conserved among flowering plants. Such studies have originated, mostly, in *Arabidopsis*. In this plant, flowering is determined by a complex genetic regulatory network consisting of a set of 'flowering-time' genes, which mediate endogenous and environmental inputs and together regulate a common subset of 'flowering integrator' genes, which in turn regulate the 'meristem identity' genes, responsible for converting a vegetative shoot to an inflorescence. Floral meristem identity genes activate or repress those downstream 'floral organ identity' genes that determine the type of organ formed from the lateral primordia (e.g., leaf versus sepal, petal, stamen or carpel), as well as genes involved in patterning the flower.[32] Among the dozens of genes that have been implicated in these pathways, a few deserve mention based on their potential as tools for studies of flowering and biotechnological manipulation of flowering in woody perennials. Photoperiodic induction culminates in the production of a small protein, called FT, which is exported from leaf cells and transported through the phloem to the shoot apex.[33] *Arabidopsis* is also promoted to flower by GAs, and the photoperiod and GA pathways converge on *LFY* and on the MADS-box gene, *SOC1*. *FT*, *LFY* and *SOC1* together regulate expression of the additional meristem identity genes *APETALA1* (*AP1*) and *TERMINAL FLOWER 1* (*TFL1*), which encodes an FT-like protein. The ability of *FT* to activate *AP1* in the incipient floral primordia depends on the bZIP transcription factor gene *FD*.[34,35] *LFY* and *AP1* collaborate within lateral meristems to promote floral development, whereas *TFL1* acts antagonistically to *LFY* and *AP1* in the primarily inflorescence meristem to maintain indeterminate growth.[36] *LFY* expression is observed at a low level before flowering, in the lateral primordia that develop into leaves, and increases in successive lateral primordia during the floral transition, and the level of *LFY* expression in lateral organs is a major determinant in the transition from shoot to floral fate.[37–40]

An immense body of literature suggests that, for the most part, these genes and mechanisms have been widely conserved among flowering plants.[41,42] Thus, they serve as a rudimentary toolbox for transgenic manipulation of flowering across a range of economically important tree fruit and nut crops, and since the work of Weigel and Nilsson in aspen, most of the remaining flowering genes from *Arabidopsis* mentioned above (or their counterparts from crop plants), have been employed in some manner. Probably the most promising of these are the FT-like genes, which have shown potential application in such diverse woody perennial as citrus,[43,22] apple,[44] plum[45] and olive.[17]

Importantly, because transgene effects tend to be dominant, plants hemizygous for flowering transgenes should flower early. The requirement for early flowering in only one parent allows breeding cycles where early flowering progeny can be recurrently selected with the desired genotype and used for the next cycle.[20,23,46] Since the transgene is no longer needed or desired in the ultimate amended cultivar, it can be eliminated by segregation in the final population. Such an approach is currently being used in combination with marker-assisted selection to incorporate resistance to several fungal and bacterial pathogens into commercial apple,[20,21,46,47] and resistance to Plum Pox Virus into plum.[23] An attractive feature of approaches that segregate transgenes is that the final genotype itself need not be transgenic. Thus, the produce might escape regulatory hurdles typically imposed on transgenics and may be more acceptable to the public.[18] On the other hand, plant transformation can lead to loss of genome integrity, associated either with somaclonal variation during tissue culture, or, where *Agrobacterium* is used, with aborted integration of the exogenous transferred DNA.[48] Such mutations might not be linked with the early flowering driver gene, and contribute an unintended trait defect to the final cultivar that is not immediately obvious. Whole-genome sequencing provides a feasible means to demonstrate that the genome of the final cultivar is intact and transgene-free.[18]

One of the most exciting potential applications for breeding involves the use of a transgenic, early-flowering genotype as a donor to promote flowering in a selected genotype through graft transmission. This strategy would exploit the potential of the FT protein to translocate, probably within the phloem stream, across a graft union. In this scenario (Figure 2), a stable transgenic line constitutively expressing FT could be used as a rootstock to drive flowering in a grafted scion, typically derived from a seedling and to be used as a pollen parent. The advantage of such an approach is that a stable, highly expressing transgenic line could potentially be used as a universal FT/florigen donor for an unrelated genotype, as long as grafts could be established. This would eliminate the need to genetically modify genotypes on a case-by-case basis.

APPLICATION OF GENOMIC TECHNOLOGIES IN HORTICULTURAL CROP BREEDING

The past two decades have seen a striking decrease in the cost and effort needed to read a plant's DNA sequence, and qualitative improvements in the infrastructure needed to store and analyze these data. These advances have provided a means both to reduce the number of cycles in horticultural breeding programs and to increase the precision and efficiency of new cultivar development. Among the most rapidly developing approaches is genome-wide selection (GWS). GWS makes use of genomic estimated breeding values (GEBVs) as selection parameters, rather than the estimated breeding values (EBVs) traditionally used by fruit breeders.[49,50] GEBVs are derived for individuals in a phenotyped training population using dense genome-wide single-nucleotide polymorphism (SNP) markers, to establish marker effects on complex phenotypes controlled by a large number of genetic loci. Individuals in breeders' selection populations are then screened and GEBVs of individuals calculated based on genetic marker information, in order to identify outstanding 'elite' individuals (Figure 3). These may then be used to advance generations, or evaluated in the field as potential cultivars.

Marker-assisted selection (MAS) for traits controlled by major genes or quantitative trait loci is now commonly employed in out-breeding woody perennial fruit crops.[51] However, MAS is now coming into its own as the key tool that renders GWS cost-effective, by enabling elimination of undesired genotypes from the original breeding population (selection population) following a relatively cheap MAS pre-screen with a few (less than 10) markers. Only those seedlings passing this filter are subjected to the much more expensive screen with the several thousand genetic markers required to enable application of GWS (Figure 3).

GWS was first established in cattle breeding programs,[52,53] then in forest trees[54,55] and field crop plants.[56,57] A recent study highlights the usefulness of GWS as a tool for tree fruit breeders, who are faced with long time intervals from seed to identification of a tree as a potential parent, as long as 7 years in apple.[50,58] This study focused on complex fruit quality traits that are commonly selected for by apple breeders, using a training population of 1120 seedlings (seven full sib families in a factorial mating design) within an ongoing scion breeding program, to ensure relevance. They found that although the majority of the 2500 SNPs individually called in the training population explained only a small proportion of trait variation, fitting all the markers simultaneously captured most of the trait heritability for a range of fruit characters. In stage II of that study, seedlings in the training population with high GEBVs provided the pollen parents for a second generation ('Selection Validation Population'), comprising 10 full-sib families. Two thousand seedlings were subjected to MAS and GWS, followed by an environmental regime developed to promote early flowering. This resulted in initial flowering of a proportion of the seedlings 27 months from seed, with full flowering expected after

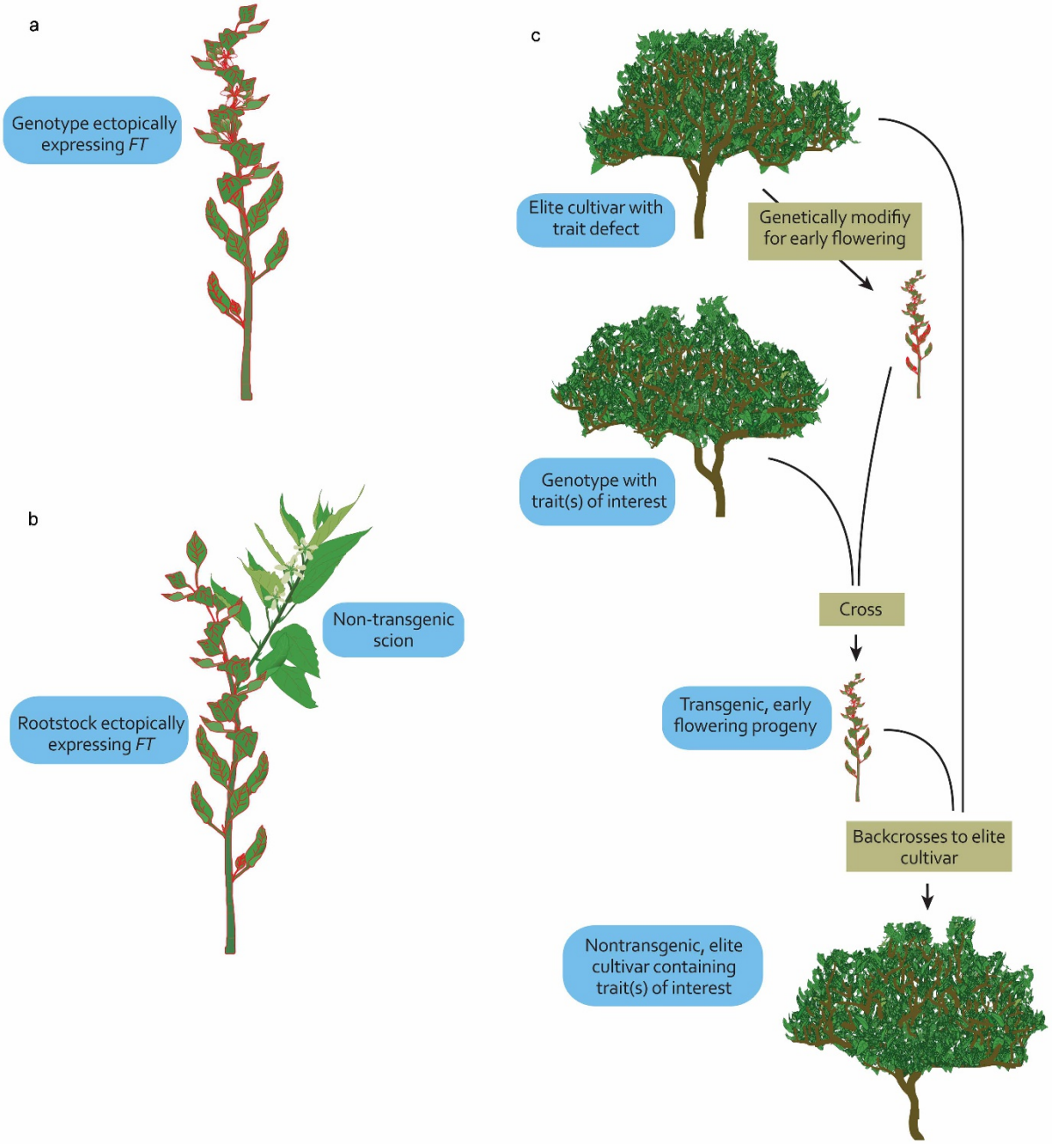

Figure 2. Approaches for accelerating the breeding cycle through biotechnology. (**a**) Flowering may be induced in a range of species through heterologous expression of one of a common subset of flowering genes, including *FT*. (**b**) The graft-transmissibility of the FT protein suggests use of a stable transgenic line constitutively expressing *FT* as a rootstock to drive flowering in a grafted scion, typically derived from a seedling and to be used as a pollen parent. (**c**) A novel approach to accelerate breeding cycle involves the use of one transgenic parent, and recurrent selection for early-flowering progeny. The transgene is eliminated by segregation after the final cross.

36 months. Fruit phenotyping will be completed in the selection population after 56 months (two assessment seasons) and fruit trait phenotypic EBVs compared with GEBVs, to analyze the observed genetic gain against the predicted gain (Volz, pers. comm.). In addition, 150 GEBV-predicted elites were clonally propagated and orchard planted for replicate testing as potential commercial cultivars 24 months from seed, 168 months (7 years) earlier than would have been achieved by conventional breeding! Based on the high accuracy of GWS in the initial study,[50] pollen from several of these elites will be used to advance to the next generation, prior to phenotyping, thus reducing the length of each cycle of breeding (Figure 3).

Many tree fruit breeding programs require thousands of seedlings to be developed and evaluated in order to make significant genetic gain across a number of important selection traits and to produce the rare individual that can be exploited as a breeding parent and/or a commercial cultivar. This poses a limitation on the application of GWS because of the relatively high cost of genotyping all individuals in such populations, currently performed with a SNP array in apple.[50,59] Continuing the practice of foreground

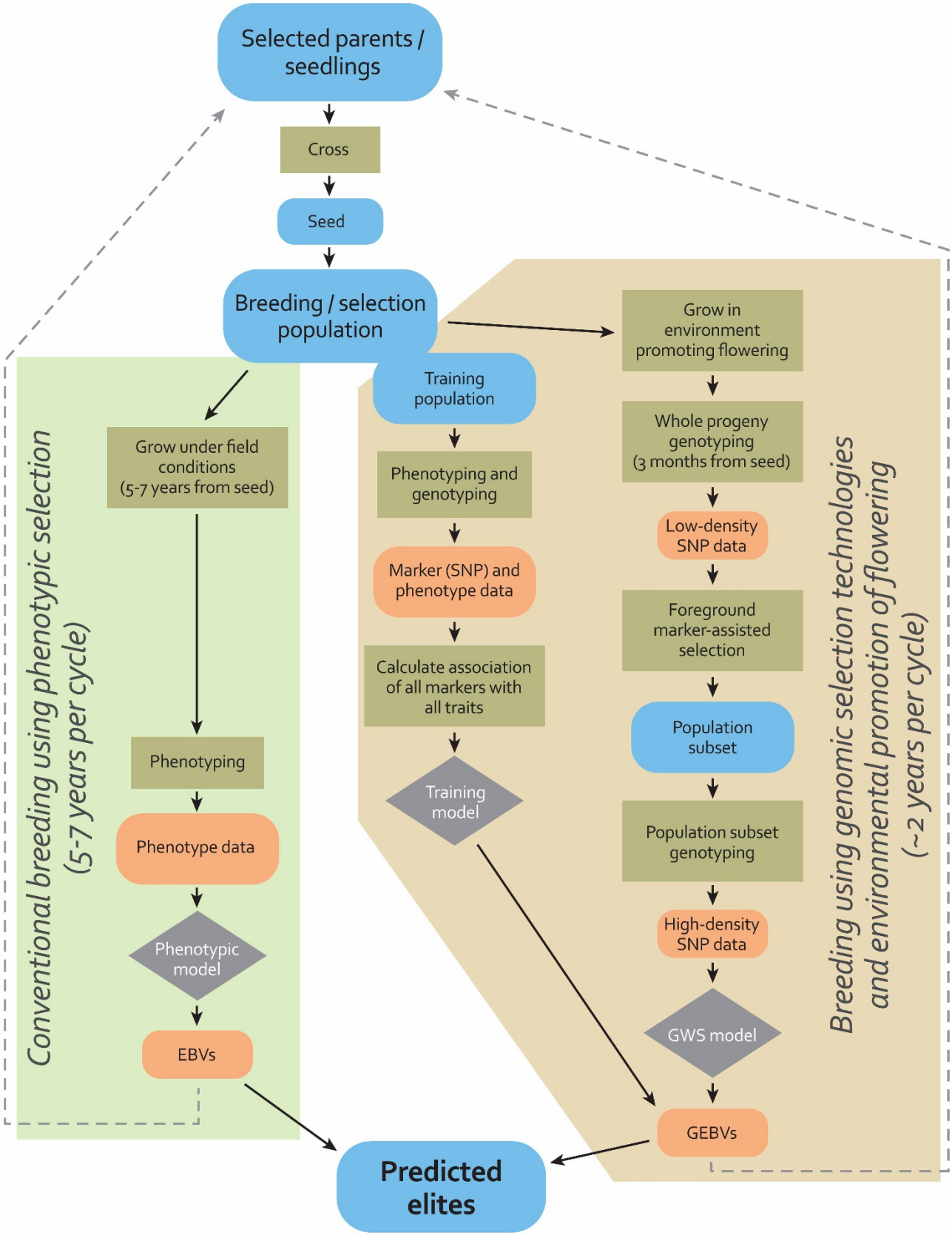

Figure 3. Comparison of apple breeding parameters between standard breeding using phenotypic selection in the field, and genomics-assisted breeding where progeny are raised in conditions that promote flowering, and foreground marker assisted selection (MAS) is applied for major gene 'must-have' traits, followed by genome wide selection (GWS) for traits controlled by multiple loci (modified from Ref. 49). In standard breeding (pathway at left), elites are selected on the basis of breeding values for fruit traits calculated from phenotypic data (EBVs). In standard breeding programs under traditional orchard conditions, phenotypic evaluation for cultivar development occurs after a minimum of 5 years from seed. This is also the earliest that elites can be advanced as parents to the next cycle of breeding and material can be further evaluated for potential as commercial cultivars. Breeding programs using genomic technologies for selection of elites (pathways at right), coupled with promotion of early flowering, can advance selected progeny to phenotypic evaluation for cultivar development as early as 2 years from seed. As flowering of the first individuals in the breeding populations is at 27 months and the remainder at 36 months, the advance of elites as parents of the next generation can occur 5 years earlier, relative to time in standard breeding programs. In effect, the length of each breeding cycle is reduced by at least 4 years (Volz, pers. comm.). Two cycles of genotyping are employed. First, MAS is used to identify plants possessing simple traits critical to the success of a cultivar (in apple this is generally pest and disease resistance). Only this subset of the original breeding population is genotyped, employing a dense marker set to enable GWS for fruit traits under more complex genetic control, thus minimizing the cost of genotyping. Genotyping for GWS currently uses an 8K SNP array, but is likely to transition to 'genotype-by-sequencing' in the near future. GEBVs are calculated from genotypic data exclusively. The application of GWS relies on the use of a training population for development of the model for association of genetic markers with phenotypic traits and this population must be both genotyped and phenotyped. It should be genetically closely related to the selection population, and in practice the model is cyclically redeveloped following phenotyping of each generation of progeny. EBV, estimated breeding value.

MAS in a breeding population for simple 'must-have traits', such as pest and disease resistances, as well as flesh or skin color, and rootstock dwarfing ability in apple, or gender in dioecious crops such as kiwifruit and hops, will enable a substantial reduction in the number of seedlings to be genotyped with dense markers for GWS into the future.[19] Another issue associated with genotyping using SNP arrays is the constraint posed by the number of SNPs on the array, which limits the application of genome-wide association studies for association of candidate markers with trait-specific alleles that can be used for screening. This limitation is being addressed by the development of arrays with higher numbers of SNPs;[60] however, this also increases the expense of screening. A step change in throughput is offered by genotyping by sequencing (GBS).[61] Its adoption in horticultural crops is rapid, with a report of its use for genetic map construction in *Rubus*,[62] as well as several conference reports on its development and application in apple,[63–65] grape[66] as well as pear and kiwifruit.[67]

In kiwifruit, *Actinidia chinensis*, GBS was employed to construct a genetic map (Hilario, pers. comm.) that was used to anchor a draft genome assembly and also trialed as speedy means of identifying genetic markers for resistance to *Pseudomonas syringae* pv. *actinidiae* in *A. chinensis*.[19] It was concluded that use of this pipeline could at least halve the time taken for future genetic marker development in kiwifruit, by enabling the simultaneous screening of several thousand markers over population phenotype extremes, compared with the previous requirement to screen genome-wide markers individually.[68] However, improvements need to be made in the area of genomic data analysis, as reduced-representation sequencing has a degree of technical uncertainty that can result in uneven sequencing of samples and consequently missing data issues in GBS analysis. A phenotyped training population developed for GWS is currently being genotyped for kiwifruit (Datson, pers. comm.).

Subsequent to the development of a draft genome assembly for European pear (*Pyrus communis*) (Chagné *et al*., in prep.), a training population was developed for the establishment of GWS in pear, using germplasm from an interspecific breeding program. This will be genotyped by the end of 2014 using GBS (Kumar *et al*., pers. comm.). A population of 1000 plants from a raspberry (*Rubus idaeus*) breeding population already phenotyped for yield and quality components is a candidate for development of GWS in raspberry (Buck, pers. comm.)

It is clear that genomic technologies will speed the development of new cultivars of a range of fruit crops, affecting the process of selecting genetically elite progeny, both for development as potential new commercial cultivars and for use as parents in the next stage of breeding. Next-generation sequencing has enabled GBS, an increasingly cost-effective means of screening individuals with large number of SNP markers densely distributed over the genome, for the application of GWS. In addition, the same technologies can be employed to identify allele-specific markers for application of foreground MAS prior to GWS.[58] Such endeavors require close collaboration by researchers from a range of disciplines, as the prime prerequisite both for training of a GWS model and for genome-wide association studies is reliable phenotyping.

PERSPECTIVES

Breeding of woody perennial tree fruit crops is transitioning from lengthy, brute-force approaches, where large populations are generated, maintained for many years under field conditions, and finally evaluated purely by phenotype, to highly intensive and selective approaches involving the genomics-assisted identification of valuable germplasm, and cycling through generations as quickly as possible. To be most effective in rapid breeding, further advancement of genomics technologies and genetic maps should be accompanied by further reduction in generation time, and *vice versa*. The interdependence justifies prioritizing rapid-cycle breeding as an integrative study drawing not only on breeding, genomics and biotechnology, but also on development, physiology and molecular genetics.

An area with exceptional promise is the transgenic manipulation of juvenility and phase change. In woody perennial plants, seasonal flowering is superimposed on phase, such that flowering occurs at the appropriate time of the season only once the plant has transitioned to the adult phase. Current efforts to drive flowering in transgenic plants exploit genes that act relatively late in the flowering pathway, and thus, bypass the juvenile phase. Studies in *Arabidopsis*, maize, and a collection of woody perennial plants[69–74] (van Nocker, 2014, unpublished data) have suggested an evolutionarily conserved mechanism, where seedling growth is accompanied by increasing expression of transcription factor genes of the *SPL* class, driven by developmental downregulation of miR156, a microRNA that targets specific *SPL* genes for degradation. A universal method for biotechnological promotion of flowering through minimizing the juvenile phase, rather than bypassing it, might allow endogenous flowering pathways to be initiated across a broad range of species, without the potential deleterious side effects associated with expression of exogenous floral transgenes.[17]

Another remaining challenge is to refine and improve approaches for biotechnological manipulation of horticultural crops. Currently, most transformation is based on *Agrobacterium*, and this technology has evolved only slowly over the past 30 years. This approach suffers from the inability to control where transgenes are integrated, leading to positional effect variation in transgene expression, as well as potential disruption of beneficial genes or chromosomal structure elements.[48] Targeted genome engineering approaches, such as the CRISPR/Cas system,[75] offer a solution,

especially if implemented independently of *Agrobacterium*-based transformation. Additional hurdles include avoiding the pleiotropy that often accompanies ectopic expression of endogenous or exogenous transgenes, due to neomorphic activity of the transgene. To overcome this, it will be essential to develop simple, genus-level crop models for molecular genetic studies. The ability to modify gene expression precisely in plants will be increasingly enabled by higher resolution mapping and annotation of genomes. This in turn will be facilitated by understanding the dynamic interaction between information encoded by DNA sequence and the 'epigenetic' information content inherent in chromatin.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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