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The Geneva Apple Rootstock Breeding Program

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ABSTRACT

The ancient practice of clonal propagation of perennial fruit crops by means of grafting was transformed when humans realized that certain properties of selected root systems could be beneficial for increasing productivity of that fruit crop. This is the case of certain clonal apple rootstocks, which were recognized for their ability to dwarf apple trees and increase the fruit/wood ratio produced by the same trees. Increased understanding of how dwarfing rootstocks can be used in orchard production systems has shaped the notion that rootstocks are the foundation of a healthy and productive apple orchard, and that as the interface between the scion and the soil, and by providing vital elements such as anchorage, water, nutrients, and disease protection, they ultimately affect the sustainability of the orchard. Therefore, it was realized that breeding of new productive, disease-resistant, dwarfing apple rootstocks using modern

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selection techniques could have positive impact on apple production by increasing orchard productivity, which in turn translates into increased profit margins for growers. Cornell University's Geneva® Apple Rootstock Breeding program initiated in 1968 is one of a handful still in activity out of a dozen initiated in the same period. What made this breeding program unique in the world was the focus on disease resistance to biotic stresses common in northeast America such as fire blight (*Erwinia amylovora*) and soil-borne diseases common to all apple growing regions such as crown and root rot (*Phytophthora* spp.), which was fueled by the vast genetic resources available in Geneva, NY. In 1998, the apple rootstock breeding program was converted to a joint breeding program with the U.S. Department of Agriculture (USDA) with a USDA breeder as the lead scientist. Breeding apple rootstocks is a lengthy and resource-intensive endeavor (research occurs in greenhouse, laboratory, nursery, and orchards) that features a multistage 20–30-year breeding process that starts with a large number of seeds from planned crosses and is whittled down to a handful of "elite selections" by multiple rounds of inoculation with diseases, the application of molecular markers for important orchard traits, and multiple evaluations in replicated orchard experiments of annual data for yield, yield efficiency, tree vigor, suckering, nutrient uptake efficiency, and response to any other unique stress events. The breeding program applies genomic and bioinformatic tools for marker-assisted breeding of apple rootstocks while leveraging discoveries in whole tree phenomics and metabolomics. Elite selections are increased to perform highly replicated tests including exposure to extreme soil temperatures, replant soils, multiple strains of fire blight, wooly apple aphids, crown rot, viruses, and graft union strength with multiple scion varieties. At the same time, the elite rootstocks are also distributed to cooperating commercial nurseries to bulk up production and evaluate propagation potential in preparation for nationwide and worldwide evaluation. During this stage, a particular rootstock may be tested in 30+ different locations and for 7–12 years at each location providing about 200 combined years of replicated data. The best elite selections are then increased in production for commercial level tests (large orchards, few rootstocks), followed by intellectual property protection and final release to nurseries and orchard growers. For most Geneva® apple rootstocks released so far, this multistage process has taken on average more than 30 years. In addition to advances in whole tree physiology, apple rootstock genomics, transcriptomics, and phenomics, the ongoing 40+-year effort in apple rootstock breeding described below has yielded a dozen of disease-resistant and productive apple rootstock varieties that are being deployed in all apple growing regions of the world. These new rootstocks are allowing growers to plant where fire blight and replant disease pressure made it virtually impossible to plant new apple orchards.

KEYWORDS: disease resistance; dwarfing; fire blight; *Malus* × *domestica*; marker-assisted breeding; mineral nutrient concentration; replant disease; woolly apple aphid

I. HISTORY OF APPLE ROOTSTOCKS

Commercial apple trees result from the union by means of grafting of two or more different genotypes: the scion (the aboveground portion that yields the fruit) and the rootstock (the belowground portion that serves as an interface between the scion and the soil). In some cases, the addition of an interstock (interstem) to the traditional rootstock–scion combination is necessary to optimize performance of the whole composite tree (Cummins 1973; Carlson and Oh 1975). Clonal propagation of desirable scions by grafting onto root systems is a technology that spans several millennia often associated with domestication and propagation of productive, delectable genotypes onto unrelated root systems (Janick 2005). The selection of rootstocks that impart particular architectural and production properties to the scion, however, is only centuries old (Tukey 1964; Rom and Carlson 1987; Webster 2003; Webster and Wertheim 2003). One can imagine fruit fanciers (including aristocrats, monks, and wealthy bourgeois) in the 17th century selecting interesting phenotypes and testing different propagation techniques and combinations on their unique germplasm (du Monceau 1768). It is probably in this way that dwarfing and precocious rootstocks were initially discovered for apples (Loudon 1822)—the most famous one being Malling 9 (M.9) derived from a population of apple rootstocks called English or French Paradise or Doucin Stock already recognized by the 1800s (Lindley 1828) and characterized in the early 1900s by scientists at the East Malling Research Station in the United Kingdom (Hatton 1917). It is likely that the "Paradise" rootstocks were initially selected not primarily for their enhancing properties (dwarfing and precocity) as rootstocks (Lauri et al. 2006) but because on their own roots the trees are dwarf, precocious, and produce early season edible fruit—an apple tree that could be grown in small quarters and gardens (Rivers 1866). Dwarfing and/or precocity were the main characteristics that led to the initial selection of these rootstocks, and then in the early and mid-20th century rootstock breeding began to address adaptation to specific biopedoclimatic conditions in apple growing regions around the world. The first breeding efforts were directed to develop rootstocks resistant to wooly apple aphids—a huge problem in the southern dominions of the British Empire—and were followed in other countries (Germany, Russia, Sweden, Poland, and Czechoslovakia) to address other weaknesses found in the Malling series of rootstocks—especially cold hardiness (Wertheim 1998). In the late 20th century, as the benefits of planting higher density, precocious, productive orchards became more evident and adopted by orchardists, several other breeding programs were initiated in several countries (Russia, Germany, Poland,

Czechoslovakia, Japan, Canada, and the United States) to address other weaknesses such as susceptibility to fire blight and Phytophthora root rot (Cornell-Geneva). The Malling series of rootstocks is the founding germplasm for all apple rootstock breeding programs as the unique source of dwarfing/precocity. When such germplasm was not used in breeding projects, what resulted was mostly vigorous unproductive rootstocks that were rarely adopted by the industry. The genetic relationship of the founding apple rootstock germplasm can be described as very restricted considering that the initial Malling series was likely interrelated and that most breeding programs utilized other domesticated apple cultivars as other parental sources—a snapshot of the diversity of commercial apple rootstocks conducted in-house using microsatellite analysis (Fig. 8.1) and other studies support this view (Oraguzie et al. 2005; Gharghani et al. 2009). Few breeding programs used interspecific crosses to overcome the weaknesses of the Malling germplasm (Fischer et al. 2000). The Cornell-Geneva breeding program was unique in the methodical search for germplasm that would complement the weaknesses of the Malling germplasm and systematically crossed such germplasm with all available dwarfing, precocious rootstock germplasm available to the program (Gardner et al. 1980a, b; Cummins et al. 1983). In this process, the Cornell-Geneva breeding program identified several *Malus* species accessions that were resistant to fire blight and Phytophthora root rot, and broadened the genetic diversity of apple rootstock germplasm that can be associated with the increase in phenotypic diversity of traits imparted by rootstocks to the scion. It is this wealth of germplasm diversity that the Geneva breeding program has enjoyed and capitalized on for producing high-performance apple rootstocks (Aldwinckle et al. 1976; Aldwinckle and Lamb 1978; Cummins et al. 1983). In recent times, breeding and development of new apple rootstocks was begun in New Zealand (Bus et al. 2008; Pilcher et al. 2008; Celton et al. 2009), in several research stations in China (Gao et al. 2011; Han et al. 2011; Rong et al. 2011; Sha et al. 2011; Yang et al. 2011; Zhang et al. 2011), and revitalized in East Malling (Webster et al. 2001; Evans et al. 2011) and Poland (Zurawicz et al. 2011; Piestrzeniewicz et al. 2013).

II. TRAITS RELEVANT FOR THE SELECTION OF IMPROVED APPLE ROOTSTOCKS

A. Tree Size Control

The canopy dwarfing trait (Fig. 8.2) has substantially reduced injuries (mainly falls from ladders) and expense in apple orchard systems. In the

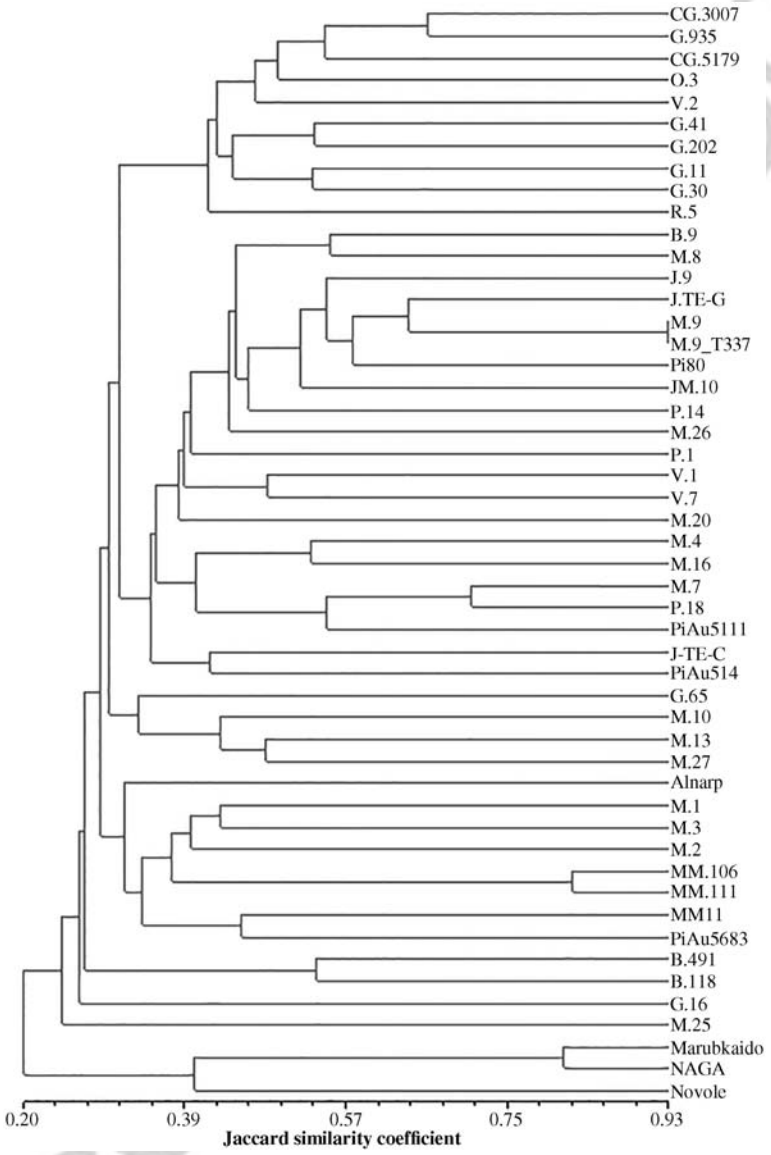

Fig. 8.1. Genetic similarity of apple rootstocks. This tree was generated with NTSYS 2.2 software for phylogenetic analysis (Exeter Software, Setauket, NY) from microsatellite data for more than 30 loci using the Jaccard genetic distance method and SAHN clustering UPGMA (unweighted pair group method with arithmetic average) routine. One can modify the input loci to represent selected loci that are linked to QTLs, perhaps giving a more functional grouping. (See color version of this figure in the color plates section.)

Fig. 8.2. The effect of dwarfing rootstocks on the size of a mature tree can be visualized in this picture juxtaposing two trees grafted with 'Mutsu' scion on different rootstocks possessing different dwarfing abilities and planted at the same time. The picture taken during the sixth leaf of a rootstock evaluation orchard in Geneva, NY illustrates a super-dwarfing rootstock's ability to dwarf the scion and shift resources to fruit production.

early 20th century, a series of apple rootstocks were collected from across Europe and characterized at the East Malling (UK) Research Station for reduced scion vigor, thereby dwarfing the apple tree without loss of productivity. The reduction of vertical size of the orchard caused by dwarfing rootstocks has reduced ladder-associated injuries and labor costs associated with harvest and pruning, and has recently enabled the mechanization of many orchard operations (Robinson et al. 2011b). The reduction in production costs spurred by dwarfing rootstocks is paralleled by the increased per-acre productivity and the more efficient use of pesticides (e.g., smaller canopy to treat) (Palmer et al. 1989; Ferree 1992; Tustin and Cashmore 1994). The ability to increase effective light interception and partition biomass production in favor of fruit instead of vegetative tissue are some of the dwarfing rootstock-based enhancements to orchard production systems that have led to significantly improved productivity per unit area (Tustin and Van Hooijdonk 2013). Most of the apple orchards planted nowadays are a testament to a transformation that has occurred in the past 60 years utilizing this centuries old technology culminating in the almost total adoption of dwarfing and precocious rootstocks. A survey of U.S. apple nurseries has indicated that 92% of the 12–17 million apple trees planted each year in

the United States are on dwarfing rootstocks. It is difficult to quantify the economic impact that dwarfing rootstocks have had on apple production but a very rough estimate made by extrapolating the difference in per-acre productivity between low-density and high-density orchards and adjusting for changes in inputs quantifies the impact between $500 million and $1.2 billion per year. Most of the dwarfing apple rootstocks in commercial use derive their lineage and dwarfing character from 'Malling 9' (M.9) and 'Malling 8' (M.8) (Fig. 8.3) (Webster 2003).

Several models have been proposed for the dwarfing trait including hydraulic conductivity (Atkinson et al. 2003; Cohen et al. 2007), plant hormone translocation (Kamboj et al. 1998; Tworkoski and Miller 2007; van Hooijdonk et al. 2011), and bud union anatomy (Soumelidou et al. 1994). Jensen et al. (2003) in an experiment comparing scion transcriptomes on dwarfing and semidwarfing rootstocks identified several genes that may be implicated in the dwarfing phenotype (Jensen et al. 2010); similarly, key flowering genes that may contribute to the shunting of resources into fruit production at a cost to vegetative growth are up-regulated in the vasculature of dwarfing rootstocks (Foster et al. 2014). Within the Geneva apple rootstock breeding program, this trait has been shown to be highly heritable, and is easily recovered in rootstock breeding populations derived from M.9, M.8, and their relatives (Fazio and Mazzola 2004). Genetic mapping using breeding populations in Geneva, NY has independently confirmed the effect and location of *Dw1*, a dwarfing locus found on chromosome 5 of the apple genome derived from M.9 (Rusholme et al. 2004; Pilcher et al. 2008). The inheritance of one locus, however, cannot explain the continuous range of vigor control observed in breeding populations. An additional locus that affects apple tree vigor located on chromosome 11 of the apple genome has been identified (*Dw2*). A multiple quantitative trait locus model (MQM) analysis that included segregating tetra-allelic markers at *Dw1* and *Dw2* loci explained 56% of the phenotypic variation in tree diameters of 5-year-old 'Gala' scion grafted onto a rootstock breeding population. The effects of these loci on tree vigor were validated in different breeding populations grafted with 'Golden Supreme' and a predictive model based on segregation of alleles at these loci was statistically significant ($P < 0.0001$) with an R^2 of 0.80 (Fazio et al. 2014b). The predictive power of such a model, however, was better at homozygous levels than when loci were in a heterozygous state; in other words, the residuals were higher in heterozygous genotypes. The interaction between the dwarfing loci was also significant ($P < 0.0001$), indicating that the effects of one were modulated by the effects of the other. When combined in different ways, these two major factors give a range of dwarfing potential that is

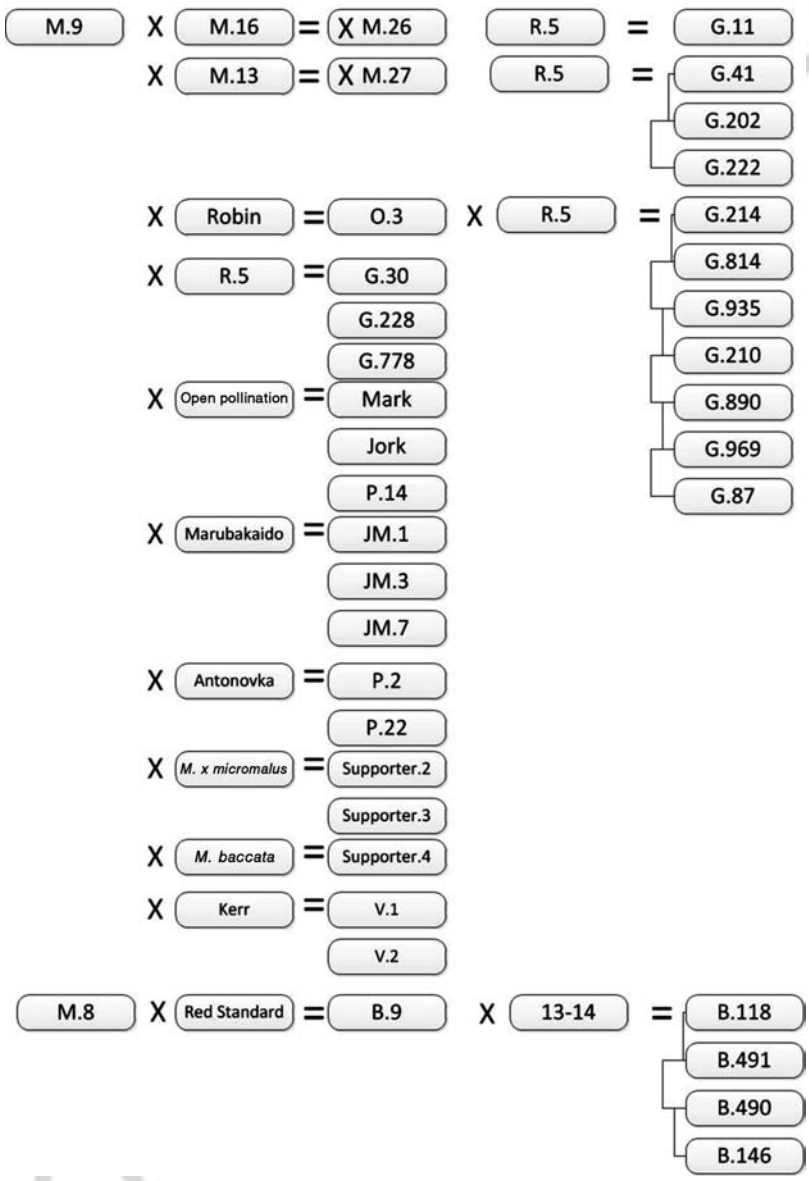

Fig. 8.3. Pedigree map illustrating the relatedness and derivatives of the most commonly used sources of the dwarfing rootstock effect: M.9 and M.8.

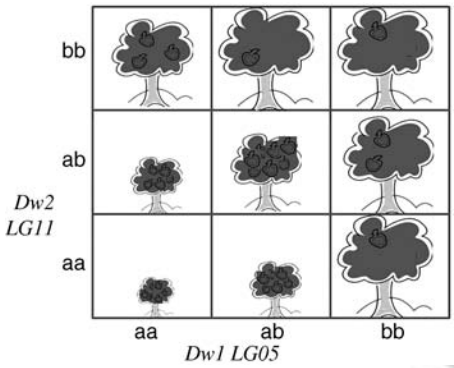

Fig. 8.4. Diagram representing the effects of the interaction of *Dw1* and *Dw2* on apple tree vigor based on least square means described in Fazio et al. (2014b). Superdwarfing rootstocks are in the lower left square. Dwarfing rootstocks are in the middle lower or middle left square. Semidwarfing to different degrees are in the other squares.

more in line with what is observed in rootstock breeding populations (Fig. 8.4). It is important to note that while the dwarfing genes can have a dramatic effect, other genetic factors relating to nutrition, biotic and abiotic stresses, and interactions with scions will have an effect on tree vigor, contributing to the overall size and productivity of apple trees. How the dwarfing loci interact with the whole plant physiology and other growth traits (Stoeckli et al. 2008) of the apple tree is not yet fully understood.

B. Induction of Feathers and Flat Branch Angles by Rootstocks

Early yield of the orchard is dependent on tree caliper, tree height, and the number of lateral branches (feathers) (Robinson and Lakso 1991; Warner 1991; Theron et al. 2000). Apple tree nurseries rely on good horticultural practices to produce high-quality trees and genotypic variation for components of tree quality characteristics (number of branches and branch angles) especially for modern orchard systems such as the Super Spindle or the Tall Spindle (Weber et al. 2000; Robinson et al. 2011b). Some intensive orchard systems similar to the Tall Spindle rely on planting a bench graft and training the scion in place as a 3 m whip in the first year of growth, followed by induction of small lateral branches to maximize early yield. These types of orchard systems place a burden onto the early performance of the rootstock that is required to push vegetative growth and then also induce flowering

and early bearing. Certain Geneva apple rootstocks have been shown to increase lateral branch initiation and branch crotch angles in commercial nurseries in Washington and Delaware (Fazio and Robinson 2008a, b). This phenomenon continues to be displayed in orchard settings and is particularly frequent in progeny of *Malus*×*robusta* ('Robusta 5') that differs from typical *M.*×*robusta* by having a wider spreading habit (Jefferson 1970). While several million trees exhibiting this new phenotype (grafted on G.935 and G.41 rootstocks that possess these abilities) are currently being planted, the mechanism underlying these traits is still not genetically and physiologically understood. Understanding, breeding, and selecting for these scion-modifying traits can have dramatic effects on the production potential of future apple orchards.

C. Induction of Early Bearing in Scions

The ability to induce early flowering in scion cultivars, otherwise known as precocity, is a major selection criterion in apple rootstock breeding because of the potential to bring an orchard to bear early and provide a quicker return on the investment to the farmer (Cummins et al. 1995). While in the literature there is an enormous amount of research dedicated to comparative phenotyping of precocity induction of apple rootstocks (Marini et al. 2006a, 2006b; Autio et al. 2011b, 2011c), articles dealing with the inheritance and possible mechanisms relating to early flower induction are nonexistent. Research in the Geneva rootstock breeding program demonstrated that two loci interactively and additively increase early flowering in an apple rootstock breeding population (Fazio et al. 2014b). These observations have been validated in other breeding populations within the breeding program and have been incorporated in the marker-assisted breeding (MAB) pipeline as this trait requires about 8 years of field observation from the time the seeds are planted for proper evaluation (Fazio et al. 2011). While other genetic sources of rootstock-induced early bearing may exist in the apple germplasm that may not share the same mechanisms displayed by M.9 and M.8, these sources will remain unidentified until replicated experiments using either core collections and/or wild germplasm (Aldwinckle et al. 1999; Volk et al. 2008a, 2014) are interrogated by grafting a non-early bearing cultivar onto diverse root systems.

D. Root Architecture and Morphology

Analysis of Geneva breeding populations has also shown that there is ample genetic variation for root architecture traits of apple rootstocks. It

Fig. 8.5. Different root morphologies observed in sibling replicates of an apple rootstock breeding population. Variation in root morphology may have an effect on soil exploration indices and overall productivity of the tree.

is possible that such traits may influence overall tree size and productivity by modulating nutrition and root/shoot ratios (Atkinson 1980). An interesting trait that we have observed in several elite Geneva apple rootstocks is the "fine root" trait, characterized by an abundance of branching of the root system that increases the exploration ability of the soil profile by the rootstock (Fig. 8.5). The genetic factors influencing this trait were mapped to loci segregating on chromosomes 4 and 11 of the apple genome (Fazio et al. 2009b). Genetic variation for root architecture in apple was also observed in seedlings of *Malus sieversii* (Fazio et al. 2009a), the wild progenitor of cultivated apples (Richards et al. 2009). An investigation that measured tree and root architecture of 500 *M. sieversii* seedlings showed correlation between canopy volume/tree size and number of thick roots was 0.38 ($P < 0.001$), and a less pronounced correlation between tree size and root mass (0.25, $P < 0.001$), suggesting that vigor of young trees may be determined in part by their ability to produce root systems with strong primary hierarchy, or vice versa by the

ability of the canopy to support the growth and expansion of a larger primary root system (Fazio et al. 2014a). Apple root systems may also vary in seasonal growth patterns (Eissenstat et al. 2006), which may affect their ability to forage for nutrients, water, and even colonization with beneficial mycorrhizae (Resendes et al. 2008). Root turnover rates may also play a significant role in tree nutrition and productivity as well as disease resistance or evasion as demonstrated by experiments that utilized replant-tolerant rootstocks from the Geneva breeding program (Atucha et al. 2014; Emmett et al. 2014). While these root traits can be targeted for marker-assisted breeding, the understanding of genes, gene expression patterns, and physiological attributes associated with these traits is limited compared with our understating of scion traits; therefore, more research is needed to understand these traits before they become the subject of selection based on genetic markers.

E. Nursery and Propagation Traits

Clonal apple rootstocks are mainly propagated by layer or stool cuttings (Adams 2010), and seldom by soft or hardwood cuttings (Bassuk and Howard 1980). Breeding for nursery performance can be quite complicated as many factors influence apple rootstock performance in the different nursery phases and at times may conflict with field performance. A prime example of this is the fast and easy adventitious rooting trait, highly desired in the propagation phase but correlated with the development of burr knots in the orchard—a harmful trait in certain orchard environments especially where dogwood borers and other insect borers may be present (Bergh and Leskey 2003). This was a problem with several Geneva apple rootstocks that had been selected to produce clean (no burr knots) shanks and also produce few or no suckers in the orchard, both highly desired traits for orchard management. This problem was in most part resolved by optimizing cultural practices for the establishment of stool beds, utilization of different propagation techniques such as cuttings or micropropagation (Hogue and Neilsen 1991), and the treatment with plant growth regulators such as prohexadione calcium in the nursery (Adams 2010). Another example of conflicting traits is the need of rootstock nurseries to produce straight shanks and finished tree nurseries to bud upright plants, while the trait that induces wide crotch angles in the orchard also affects the architecture of the stool beds and the budded trees by opening their canopies. This problem was overcome by widening the stool bed row through the use of double-row plantings, the use of guide strings on each side of the bed, and by finished tree nurseries with modified tree training techniques.

Nursery professionals look for straight shanks, a minimum number of healthy primary roots, lack of spines, graft union strength, and scion compatibility. Most commercial apple rootstocks comply with these requirements, but when rootstocks diverge from their optimum, if the rootstock is in high demand because of its horticultural properties in the orchard, nursery professionals are usually quick to devise management solutions to overcome the weaknesses of the rootstocks. Rootstock genotype can have very dramatic effects on the characteristics and value of a nursery tree. The number of feathers and caliper of trees are two characteristics associated with planting tree quality (Fazio and Robinson 2008b; Kviklys et al. 2011). Another characteristic affected by rootstock is graft compatibility. Most problems that were blamed on compatibility turned out to be virus related (Cummins and Aldwinckle 1983); however, certain rootstock scion combinations under unspecified grafting and nursery management conditions have shown a tendency for weak graft unions in very young trees (Robinson et al. 2003). To further study the influence of rootstock genotype on graft union strength, we are currently evaluating the method of grafting (chip budding, whip and tongue grafting, and machine V grafting) on union strength at various stages in the nursery cycle and in the orchard. We theorize that the whip and tongue method of grafting provides more contact area between the scion and the rootstock and should give greater graft union strength. We are also investigating the effect of plant growth regulators on the generation of connecting tissue and graft union strength. Another factor affecting graft union strength that is under investigation is the age/size of the rootstock liner being grafted and the ability of the rootstock/scion combination to generate enough connective tissue where they meet. It seems that large caliper stocks may not form as strong of a graft union as small caliper stocks; therefore, a rootstock genotype that produces smaller caliper liners from the stool bed may be more suitable for nursery tree production. We have not been successful at evaluating finished nursery tree traits early in the breeding cycle because such traits are somewhat hard to evaluate and require high replication in order to detect rootstock effects on many different scions (Song et al. 2013).

Grafting of interstems onto a seedling or other vigorous clonal rootstock is another method of propagation (Luo et al. 2013) that is used to capitalize the dwarfing ability of rootstocks (Cummins 1973). The length of the dwarfing interstem is inversely proportional to vigor induction and proportional to the induction of early bearing in apples (Carlson and Oh 1975). While this method of propagation is still used in some apple growing regions, most nurseries have moved away from this system because it adds extra costs (labor, materials, time, and risks) to the

development of a finished tree and it is not always reliable. While rootstocks may differ in many of the traits that determine nursery quality, in practice, breeding and evaluation is performed mostly on rooting, the absence of spines, and the straightness of the shanks, taking into consideration how selection indices for those traits will affect orchard performance.

Breeding apomictic apple rootstocks has several attractive aspects in terms of nursery management since seed is more easily transported, free of viruses, and provides opportunities for adjustment in production level to match demand. While apomictic rootstock selections have been identified and evaluated, this material has not gained much traction because of inherent problems with other characteristics in this germplasm (Schmidt 1972a, 1972b; Ferree 1998) including the lack of early bearing induction so critical for modern production (Sax 1949). Breeding for apomicts still continues in certain programs in China (Sha et al. 2011; Ma et al. 2012); however, because of the difficulties related to hybridizing with apomictic species (Bisognin et al. 2009), most breeding programs have abandoned this effort.

F. Absorption of Nutrients and Translocation to the Scion

Apple rootstocks provide anchorage and access to water and nutrients to the scion. During the process of breeding new apple rootstocks, very little attention has been devoted to studying the genetic inheritance of absorption and translocation of macro- and micronutrients by the roots into the scion leaves and fruit. It has been hypothesized that increasing the efficiency and absorption of nutrients such as zinc in the rootstocks through transgenic approaches or breeding may have an effect on overall productivity of the rootstock/scion/orchard management combination (Swietlik et al. 2007). Rootstocks vary in their intrinsic genetic ability to forage for nutrients and transmit them to various organs of the scion and may in turn affect postharvest qualities of the fruit (Andziak and Tomala 2004; Fallahi 2012). Most research on the effects of apple rootstocks has focused on measurements of optimal nutrient concentration on a few rootstocks under different management systems (West and Young 1988; Vaysse et al. 2000; Chun et al. 2002; Neilsen et al. 2008; Fallahi et al. 2011; Fan and Yang 2011). Apple roots may also possess different growth patterns and absorbance efficiencies based on genotypic effects (Eissenstat et al. 2001). Other factors such as soil type and soil pH interact with rootstock genotypes to influence scion nutrient status (Fazio et al. 2012b). Quantitative trait analysis in an apple rootstock breeding population uncovered quantitative trait loci (QTLs) for leaf mineral

concentrations of potassium (K), sodium (Na), phosphorus (P), calcium (Ca), zinc (Zn), magnesium (Mg), and molybdenum (Mo) (Fazio et al. 2013). Correlation analysis of the relationships among different nutrients uncovered significant positive linear correlations between Ca and Cu, Mg, P, and S. A significant correlation was also detected between Cu and K, Cu and P, also between K and P and between S and P. Segregation of a major QTL for leaf K concentration in certain rootstocks had strong effects on the concentrations of other nutrients in the leaves (Fazio et al. 2012a). It is possible that even subtle changes in plant nutrients caused by variable gene combinations in the rootstocks can affect fruit quality/size (Hirst et al. 2000), productivity, and disease resistance of apple trees. For example, rootstocks that are more efficient absorbers and translocators of calcium may be able to mitigate postharvest disorders such as bitter pit (Val et al. 2000). The discovery, breeding, and selection of efficient absorbers and translocators of mineral nutrients should increase efficiency and predictability for developing better rootstocks and have a positive impact on sustainable apple production worldwide (Fazio et al. 2012a, 2012b). The landscape of genetic factors influencing rootstock-based scion nutrition is rather complex perhaps resulting from the multifaceted absorption and transport models and interactions between different nutrients (Fazio et al. 2013). At this point, the incorporation of nutrient-related traits into a marker-assisted breeding scheme may be possible but needs to take into account the effect that increasing/decreasing a particular nutrient concentration has on other nutrients and physiological parameters in the scion and in the finished fruit. To date, there are no published studies linking genetically transferred, rootstock-based nutrient modulation to fruit quality and tree productivity parameters, making the choice about which markers to employ nearly impossible.

G. Disease and Insect Resistance

The Geneva apple rootstock breeding program was founded to develop disease resistance to devastating diseases in North America such as fire blight and Phytophthora root rot (Cummins and Aldwinckle 1974; Cummins and Norton 1974). This focus has resulted in the development and release of breeding populations and apple rootstocks resistant to fire blight caused by *Erwinia amylovora* (Norelli et al. 2003; Fazio et al. 2005; Russo et al. 2007; Robinson et al. 2011a), crown and root rot caused by *Phytophthora cactorum* (Johnson 2000; Robinson et al. 2003), tolerance to the replant disease syndrome (Rumberger et al. 2004; Leinfelder and Merwin 2006; Auvil et al. 2011; Robinson et al. 2012), and resistance to

wooly apple aphids (*Eriosoma lanigerum*) (Johnson et al. 2001). Breeding for insect and disease resistance continues to be an important focus and should be of importance for any apple rootstock breeding program.

1. Apple Replant Disease. This syndrome includes stunting of young trees and substantial losses in production over the lifetime of the orchard (Jones and Aldwinckle 1990). It is one of the major challenges facing apple growers in the northwest of the United States as potent chemistries that were used to combat this problem (methyl bromide) have disappeared and available virgin land optimal for orchard establishment has drastically decreased leaving the options to replant in old orchard sites or planting in marginal lands (Auvil et al. 2011). Loss in production due to replant disease is becoming more of an issue in other apple producing regions of the world (Costa and Stassen 2011; Mac an tSaoir et al. 2011; Mazzola and Manici 2012; Yim et al. 2013). Removal of an old apple or pear orchard often leaves significant active biological residues adapted to scavenging and causing disease on apple (or pear) root systems. This residual presence has been shown to last several years of fallow management. When nursery trees are planted in those conditions, their young root system is overwhelmed by the presence of the residual biological activity from the past orchard's root systems and results in poor growth and productivity of the new orchard. There is also evidence that this poor growth is accompanied by a decrease in concentration of several macro- and micronutrients in the leaves of the scion (Fazio et al. 2012b) indicating that the organisms and other factors causative of replant are disrupting the ability of rootstocks to absorb and translocate important nutrients to the scion. Several causative agents have been implicated in the etiology of apple replant disease (ARD) including *Cylindrocarpon destructans*, *P. cactorum*, *Pythium* spp., *Rhizoctonia solani*, and various pathogenic nematodes (Mazzola 1998). The activity of possible causative organisms differs from site to site, probably by the influence of climatic, edaphic, and other environmental factors. Conventional treatment for this complex disease includes various forms of fumigation or drenches including methyl bromide, chloropicrin, and nematicides. Notwithstanding the environmental hazards associated with some of these treatments, their efficacy is at times poor (Yao et al. 2006) and very dependent on edaphic and other environmental conditions. Alternative treatments to fumigation that are efficacious and much friendlier to the environment are badly needed. Several of these treatments including seed meal amendments, fertilizers, compost teas, and solarization have been proposed and are in various phases of research and development (Wilson et al. 2004; Mazzola and Mullinix

2005; Braun and Fuller 2006; Mazzola et al. 2007, 2009). Planting new tree rows in a manner to avoid the location of old tree rows has shown success (Leinfelder and Merwin 2006). Practical implementation of this method is dependent on the new orchard design and architecture. A comprehensive study on genetic tolerance to ARD that included several commercial rootstocks and wild species showed a significant difference among genotypes in their ability to grow in nonpasteurized ARD soils (Isutsa and Merwin 2000). Recently, cultivar specificity in the interaction between plants and the resident rhizosphere microflora has been reported (Gu and Mazzola 2003; Mazzola 2004; Rumberger et al. 2007) with evidence suggesting that the rootstock genotype influences apple replant disease and root-zone microbial community composition in an orchard soil, such that tolerant rootstocks will not cause a virgin soil to become ARD active (St. Laurent et al. 2010). The multipartite nature of this disease, the complex interactions among its components, and the lack of knowledge about genotypic root physiology make ARD a difficult problem to unravel. Geneva® rootstocks are the only apple rootstocks reported to have tolerance to ARD. The selection protocol including a screen for *Phytophthora* spp., *Erwinia* spp., and other rootstock pathogens (Johnson 2000) may have resulted in the serendipitous selection of genes that effect tolerance to ARD as evidenced by the above-cited studies. Another possible factor that may play a role in what is perceived as general tolerance to ARD is the presence in many of the rootstocks reported to be tolerant to ARD of the fine root characteristic. In addition, the tolerance to ARD may be due to genes that increase the efficiency of uptake and translocation of key nutrients such as potassium, phosphorus, and iron, as recently discovered in breeding populations in Geneva (Fazio et al. 2012a). This would partially explain the observation by the breeder (Fazio) that certain rootstock genotypes exhibit superior performance in disparate ARD sites around the world.

2. Fire Blight. This devastating disease, which is caused by *E. amylovora*, an anaerobic, Gram-negative bacterium, causes visible symptoms in blossoms, fruits, shoots, and woody tissue of apple and pear (van der Zwet and Keil 1979; van der Zwet 1989; Jones and Aldwinckle 1990). This bacterial disease was widely spread from its first observed occurrence in the Hudson Valley of New York state throughout North America in the 1900s (Aldwinckle and Beer 1979) and has now spread to Europe and other parts of the world (Jock et al. 2002; Palacio-Bielsa et al. 2012). The rootstock phase of the disease can be very devastating as the bacteria that reach susceptible rootstocks have the ability to girdle and kill the whole tree (Aldwinckle et al. 2004). Unfortunately, as the disease

spreads, its devastating effects are compounded by the widespread use (70–85% of trees planted in 2011) of highly susceptible dwarfing rootstocks (M.9 and M.26) that are preferred over others because of their ability to increase productivity of high-density orchards. Antibiotic sprays such as streptomycin can prevent some of the phases of the disease of the scion and may have mitigating effects on the rootstock phase, but they are not an effective control of rootstock blight. In addition, antibiotic-resistant strains of *E. amylovora* are appearing in several places in the world complicating prevention of the disease (Jones et al. 1999; Russo et al. 2008; Bekoscke et al. 2014). If the rootstock had genetic resistance but the scion did not, the bacteria could still infect the aboveground portions of the tree but would not kill the tree as occurs when the rootstock is susceptible. Furthermore, the control of the aboveground disease is currently successfully managed with the use of antibiotics. The recent registration of a new antibiotic (kasugamycin, Kasumin) in the United States to be used against fire blight will help with this effort. Genetic resistance to bacterial diseases has been observed in higher plants, and in this case resistance to *E. amylovora* has been observed in wild apple species and some cultivated cultivars (Aldwinckle et al. 1976; Forsline et al. 2002). Characterization of novel sources of fire blight resistance in the apple germplasm collection of the Plant Genetic Resources Unit (Geneva, NY) has resulted in the identification of several new sources of resistance (Volk et al. 2008b; Fazio et al. 2009a) that have been incorporated into the breeding program in recent crosses. This natural resistance can be utilized to avoid costly chemical sprays and potential damage to the environment. Apple rootstocks genetically resistant to fire blight (G.65, G.11, G.16, G.30, G.202, G.41, G.935, G.214, G.969, G.890, G.222, and G.210) have been developed through conventional breeding. Several of these rootstocks derive their resistance from 'Robusta 5' (R5). The resistance derived from R5 is strain specific (Norelli et al. 1987; Fazio et al. 2006, 2008) and the inheritance has been characterized in several independent populations (Peil et al. 2007; Gardiner et al. 2012). The use of some fire blight-resistant rootstocks has been shown to decrease the severity of the disease to a limited extent in certain susceptible scion cultivars (Jensen et al. 2011, 2012) possibly by changing the expression of genes during the infection (Norelli et al. 2008, 2009; Baldo et al. 2010). The prospect for marker-assisted breeding for fire blight resistance is good only when the costs associated with it show improvement over the practicality of the inoculation treatments performed in stages 1 and 2 of the breeding project. Such inoculation treatments have proven to be the most efficient way to select resistant material in the breeding

program. Marker-assisted breeding may be valuable when seedlings need to be screened in conditions not favorable to the disease screen such as in countries where the pathogen is not present.

3. Woolly Apple Aphid. Developing rootstocks resistant to *E. lanigerum* has been an important objective early in modern apple rootstock breeding programs as the pest was prevalent in apple growing regions in the southern hemisphere (Damavandian and Pringle 2007; Shaw and Wallis 2008). Recently, however, woolly apple aphid (WAA) has become a more severe pest in Washington state (Beers et al. 2010) and an invasive pest in China (Wang et al. 2011). Milder winters have promoted overwintering survival on the belowground parts of the tree. In many parts of the world, new plantings are rarely made on resistant rootstocks, and a very low percentage of the acreage is currently on a resistant rootstock except in South Africa. The transition from organophosphate insecticides to either insect growth regulators or neonicotinyl insecticides may also be contributing to higher pest pressure (Beers et al. 2006). It may be possible to control this pest significantly by utilizing genetically resistant rootstocks in new apple plantings. These rootstocks would significantly reduce the ability of WAA to overwinter in the root zone and therefore decrease their survival and the rapidity of recolonization of the aerial portions of the tree each spring. A greenhouse test of eight clonally propagated rootstocks and two seedling rootstocks demonstrated that several of the new Geneva rootstocks have virtual immunity to a Washington state strain of WAA due to the presence of the Robusta 5-derived *Er2* WAA resistance gene located on linkage group 17 of the apple genome (Bus et al. 2008, 2010). The Geneva breeding program has released six rootstocks that take advantage of this gene (G.202, G.41, G.222, G.214, G.969, and G.890) and in case of G.202 that has been planted in high insect pressure environments in New Zealand for the past 10 years we have seen no evidence of breakdown of such resistance. This is a logical target for marker-assisted breeding as the effect of the gene is dominant and mass phenotypic evaluation is rather difficult. Haplotype-specific PCR markers have been developed and are currently deployed in the Geneva breeding program to ensure the presence of this gene in future populations and select resistant material that is in advanced stages of the breeding process (Jensen et al. 2014).

4. Tolerance to Viruses. While the ultimate goal of the apple industry should be to work with certified virus-free material tested with indicator plants (Howell et al. 1996) or other immunological, proteomic, or

genomic methods (Silva et al. 2008; Mathioudakis et al. 2010), the pervasive presence of latent viruses in nursery stocks is the current reality and poses a problem where susceptible rootstocks are used with scions that may not be virus-free. Infection by latent viruses was not a concern with the Malling rootstock series, since they showed tolerance to the latent viruses and generally had only small losses in productivity or changes in fruit quality in infected scion/rootstock combinations (Campbell 1981). However, utilization of wild species in breeding new rootstocks has introduced sensitivity to latent viruses, such as the decline caused by Apple stem grooving virus observed in 'Ottawa 3' (Wertheim 1998) and its progeny 'G.16'. Some viewed this sensitivity as a positive trait because it should force the industry to utilize clean wood during propagation; however, significant losses sometimes have occurred due to a low nondetectable virus titer that increased slowly, causing tree decline 2 or 3 years after the orchard was planted. This problem with G.16 made it clear that some segments of the industry were not ready to adopt such rootstocks with a virus sensitivity and that it would be preferable to breed rootstocks that are tolerant to viruses. Another virus issue is rootstock sensitivity to the slow decline caused by graft union necrosis between certain rootstock/scion combinations in the presence of Tomato ringspot virus (ToRSV) (Tuttle and Gotlieb 1985a, 1985b; Griesbach 1995). MM.106 rootstock grafted with Delicious scion is the classic example of this problem. To date, there is no conclusive data on the sensitivity of the Geneva® rootstocks to ToRSV-induced graft union necrosis, but a large trial is underway in New York state to evaluate 50 genotypes for this sensitivity. To this date, no genetic studies are available on the susceptibility of *Malus* germplasm to viruses. In the Geneva breeding program, virus-sensitive parents such as G.16 are being utilized for crosses, and efforts to map susceptibility loci are underway as a prerequisite to marker development to be utilized for culling susceptible seedlings before resources are wasted on growing them in trials.

III. GENERAL APPROACHES AND RESEARCH PROCEDURES FOR BREEDING NEW APPLE ROOTSTOCKS

Below we describe a general approach and procedures for the 17–25-year process to breed new apple rootstocks integrating classical and marker-assisted breeding exemplified by the Geneva breeding program. The process is also outlined in Fig. 8.6.

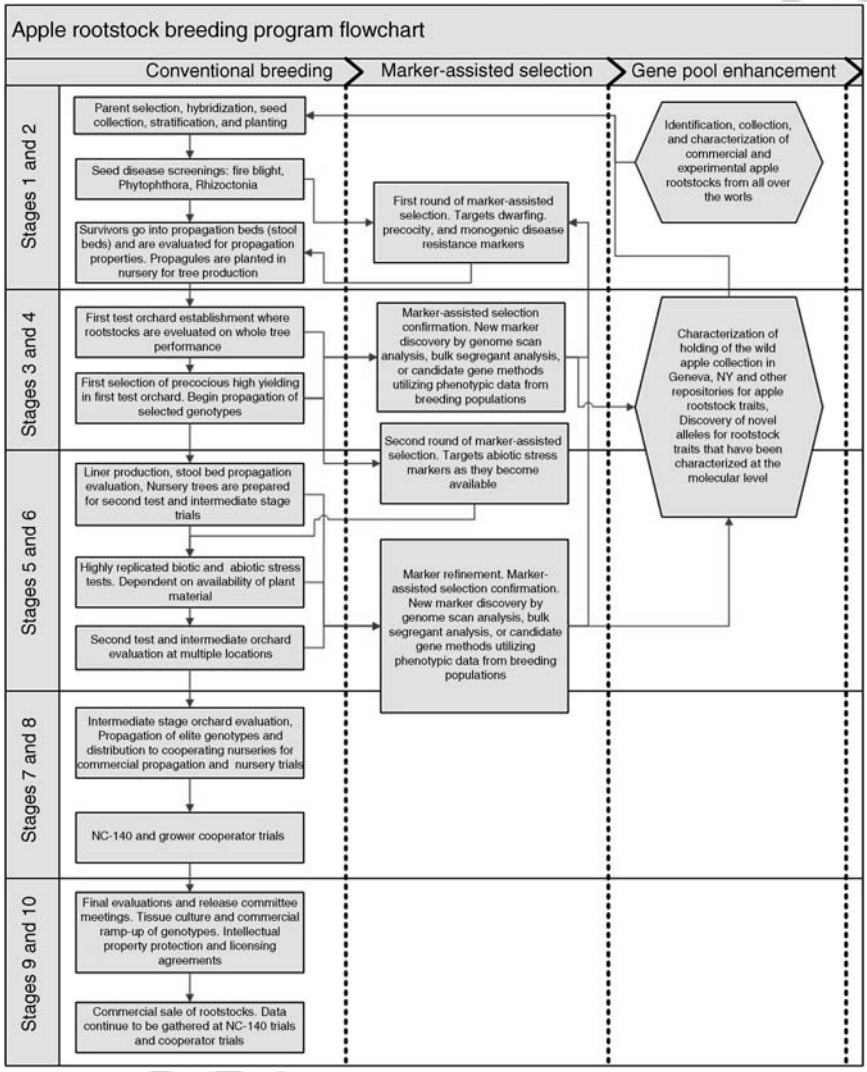

Fig. 8.6. Diagram showing different breeding stages and integration of marker information to increase efficiency.

A. Resources Needed to Breed Apple Rootstocks

Apple rootstock breeding is a resource-intensive process requiring the combination of a genomics lab, plant pathology lab, greenhouses associated with raising seedlings, performing inoculations, and propagation, as well as a stool bed nursery for the production of seedling and elite rootstocks and a finished tree nursery to produce test trees; in addition, winter root and tree processing and storage facilities are needed to prepare, trim, and evaluate rootstock liners and finished trees. All this has to be accompanied by well-managed orchard land with irrigation and plant protection against pests and related fruit harvesting, storage, and evaluation facilities.

B. Breeding Objectives and Philosophy

Clear objectives are needed in order to achieve the goal of developing superior apple rootstocks, while applying advances in marker-assisted breeding. Such objectives need to match the resources and facilities available for breeding. For example, the objective of developing WAA resistance in new apple rootstocks might require greenhouse space to rear the insects and another space for inoculation; conversely, it might also require a modern genomics laboratory for development and deployment of molecular markers that discriminate resistant from susceptible plants. The continuous identification and characterization of novel traits that are associated with increased productivity, improved propagation, and increased tolerance to biotic and abiotic stresses should be a priority and where possible the newly identified traits should be included in the breeding process. In addition, the continuous identification of novel sources of traits in the unadapted germplasm pool should be investigated and incorporated wherever possible.

Characterization of rootstock selections after the initial selection based on dwarfing, production efficiency, and resistance to diseases and insects is a major task and requires cooperation of physiologists. There are a number of physiological characteristics that would make sense to measure on rootstocks but because of labor requirements, cost, and instrument requirements, they have not been utilized to their fullest potential to characterize and understand the performance of apple rootstocks. For example, one such characteristic is the function that root systems have to absorb and translocate nutrients to the grafted scion (West and Young 1988; Vaysse et al. 2000; Chun et al. 2002; Neilsen et al. 2008; Fallahi et al. 2011; Fan and Yang 2011) and how that activity is

affected by soil type, root architecture (de Sousa et al. 2012), water relations, soil pH, and different grafted scion cultivars (weak type, vigorous, spur type, and tip bearing). This activity requires highly replicated (10+ trees/replication) pot or field experiments where trees are treated with different soil types, water regimens, soil amendments, and inoculation with pathogens, and parameters including tree growth, photosynthesis, nutrient concentration in the leaves or fruit, and tree architecture parameters (bud break, rooting, root morphology, branching, growth, and flower induction) are measured using a set of diverse but related rootstocks in the breeding program to enable genetic analysis. Other important examples are rootstock genotype cold hardiness and graft union strength between rootstock genotypes and scion genotypes. The resulting data can be used to estimate heritabilities, correlations, and the location/effect of genetic factors segregating in the breeding program. An example of such exploratory experiment is the work done within the Geneva breeding program to discover genetic variation for soil/pH and nutrient foraging adaptations (Fazio et al. 2012b), which generates potential for these types of experiments to help researchers and apple growers make more informed decisions regarding the type of rootstock that matches their edaphic–climatic conditions and discover the underpinnings of such complex traits.

C. Breeding Stages and Integration with Marker Information

Johnson et al. (2001) identified 10 stages of breeding and selection within the Geneva breeding program and they are described below with several updates and modifications based on the application of marker technologies and new traits being investigated.

1. Stage 1: Parental Selection, Hybridization, Disease Screenings, Stool Plant Establishment, Years 1–2; 2,000–10,000 Seedlings. Parental combinations that have complementary characteristics are chosen for hybridization; for example, an easily propagated dwarfing parent might be crossed with an exceptionally disease-resistant parent. Seeds are collected from the fruit of these crosses, and the seeds are stratified (cold treated to break dormancy) and germinated. Seedlings (2–3 cm tall) are inoculated with a mixture of virulent crown/collar rot oomycete pathogens (*Phytophthora* spp.) (Cummins and Aldwinckle 1974), and survivors that reach 15–20 cm in height are then inoculated with fire blight bacteria (*E. amylovora*) (Gardner et al. 1980b). The experience in Geneva has been that these two inoculations eliminate 50–80% of the seedlings. DNA is extracted from all surviving seedlings and tested for the presence

of the major dwarfing loci and other markers associated with important traits (WAA resistance, nutrition) using high-throughput PCR markers (SCARs) that have been developed in our laboratory. Rather than a generalized approach, individual apple rootstock breeding and research programs should develop their own set of molecular markers based on genomic location, parental germplasm, detection instruments, and desired throughput. Depending on the parents used, markers generally eliminate 75–95% of surviving seedlings. Selected plants are then transplanted into pots and eventually planted in the field to establish single plant stool beds—otherwise known as mother plants for all clonal propagules needed for further evaluation.

2. Stage 2: Stool Plant Selection, Nursery Liner Establishment, Nursery Tree Growth, Years 3–4; 25–100 Stool Trees. Genotypes are propagated as single tree stool plants, and nursery liners are harvested from genotypes that show adequate rooting (at least three adventitious roots per shank) and do not have brittle wood. It generally takes 2–3 years in the field to grow plants that are large enough to produce the needed number of propagules (rootstock liners) for further evaluation. Liners that pass the minimum requirements are processed in cold storage in preparation for planting, and planted into a nursery where during years 5 and 6 finished trees are produced by budding or bench grafting. In years 5 and 6, stool trees are again evaluated for resistance to fire blight and for infestation levels with wooly apple aphids (Johnson 2000), and susceptible genotypes are discarded from the nursery and from the stool beds.

3. Stage 3: First Test Orchard Establishment, Precocity Evaluation, and Selection, Years 5–6; 25–50 Rootstock Genotypes. Where marker-assisted selection for dwarfing rootstock genotypes has been implemented, the expectation is that the vast majority of rootstocks will be dwarfing allowing for higher densities in the first test orchard and perhaps training with more modern spindle methods. This stage usually requires 4–10 finished trees on each rootstock genotype planted in a completely randomized fashion. In addition to the new rootstock selections, standard rootstock genotypes are included (G.41, G.935, G.210, M.27, M.9, and B.9) to allow an initial estimate of vigor control and precocity induction of the rootstocks. Trees are trained to a central leader tree type, but pruning is kept to a minimum to allow the natural rootstock-induced architecture to express itself. In certain situations, pruning may be performed to pattern a slender or tall spindle system. Data are collected annually on each individual tree for yield (number and weight of apples), yield efficiency, tree vigor, suckering, nutrient uptake

efficiency, and response to any unique stress events. Statistical analysis of these data is a straightforward one-way ANOVA with rootstock genotypes as the main factor.

4. Stage 4: Elite Stool Bed Establishment, Years 7–12; 10–15 Rootstock Genotypes. Rootstock genotypes that exhibit precocity and adequate yield efficiency by the fourth leaf (year 10 of breeding cycle) are propagated to increase plant material for an elite stool bed in a second location. Stool beds are developed from liners retained from stage 2 or from root cuttings of older orchard trees.

5. Stage 5: Liner Production, Stool Bed Evaluation, Nursery Tree Growth, Years 10–15; 5–10 Rootstock Genotypes. The important factor of this stage is development of enough clonal propagules (liners) to be able to run highly replicated tests and produce a reliable estimate of how resistant or tolerant a selection is to different biotic and abiotic stresses that the new rootstocks will ~~be faced~~ within the life of an orchard. This critical number of plants per genotype is between 100 and 1,000 and is very difficult to achieve with conventional propagation methods and may be best achieved through micropropagation. At this stage, trees are produced with several scion/rootstock combinations to test graft union compatibility and strength. Liners in the nursery are budded with 'Golden Delicious' or 'Gala' (an easy to grow, familiar cultivar with wide adaptation) to produce 30 high-quality finished trees. First test orchards established during stage 3 are removed after harvest in year 15 (after ninth leaf). After 30 trees are produced in the nursery, liners are collected from elite stool beds and subjected to evaluations of disease resistance and stress tolerance, extreme temperature soil tests (trees are grown in heated pots), early and mid-winter cold stress (Moran et al. 2011), replant soil tests (Isutsa and Merwin 2000), fire blight tests, crown rot tests, virus resistance/hypersensitivity tests, and graft union strength tests (3–4-year-old finished trees with several scion/rootstock combinations are subjected to mechanical stress at the graft union) while in stages 5–7 (Johnson 2000). Several protocols and methods for these techniques have been described, but need to be adapted to local conditions (Cummins and Aldwinckle 1974).

6. Stage 6: Intermediate Stage Orchard Establishment and Early Evaluation, Years 16–18; 10 Rootstock Genotypes. Intermediate stage orchards are planted beginning in year 16 at three sites representing a cross section of domestic/international apple production environments.

Each year's planting includes commercial standard dwarfing genotypes (M.9, B.9, and M.26) and 5–10 elite rootstock genotypes that have shown promise in elite stool bed liner production, initial test orchard performance, and biotic and abiotic stress resistance screens. These orchard trials use a replicated experimental design with ~10 individual tree replicates and are evaluated for precocity in their early years and production efficiency over an 8–10-year period.

7. Stage 7: Commercial Stool Bed Trials, Years 19–21; 5 Rootstock Genotypes. Intermediate stage orchard trial data collection continues. Biotic and abiotic stress screenings of rootstock liner trees are completed. The most promising rootstock genotypes are distributed to cooperating nurseries for commercial stool bed trials beginning in year 19. The most promising rootstock genotypes are submitted for phytosanitary certification to enable international distribution. The transfer of plant material (rootstock liners) to cooperating nurseries is important as these are the entities that will propagate the material for future use by the industry; these commercial trials allow the nurseries to gain familiarity with the material to generate finished trees. Data on larger scale nursery tree performance (transplanting success, budding success) are collected at this stage. This first look by cooperating nurseries allows the nurseries to learn the best cultural practices useful with the new genotypes. For example, some genotypes may differ in application timing and requirements of mulching compounds or plant growth regulators to increase rooting and overall quality of the liners.

8. Stage 8: NC-140 and On-Farm Trials, Distribution to All Cooperators, Years 22–24; 2–5 Rootstock Genotypes. Intermediate stage orchard trial data collection continues (Robinson et al. 2006). For outstanding rootstock genotypes from the intermediate stage orchard trials and commercial nursery stool bed trials, liner production from cooperating nurseries is used to propagate trees for NC-140 (Autio et al. 2011a, 2013) and/or on-farm trials. Each multistate NC-140 trial and on-farm grower cooperator trial is unique and follows methods and protocols that are established by the cooperators participating in the trial. Generally, data on survival, tree size, yield efficiency, productivity, precocity, hardiness, and incidence of disease are collected for each rootstock for a period of 8–10 years. ~~Best~~ rootstock genotypes are distributed to domestic cooperating nurseries for propagation, and to international cooperating nurseries and institutions for propagation and local evaluation trials.

9. Stage 9: Commercial Ramp-Up, Patent Applications, Years 25–27; 1–3 Rootstock Genotypes. Plant material of rootstock genotypes demonstrating marked improvement over commercially available cultivars based on results from cooperator and NC-140 trials increases in commercial stool beds and micropropagation facilities. Applications for plant patents and plant breeder's rights are filed on commercially viable rootstock genotypes to facilitate the successful transfer of the technology to the industry. This step may seem trivial but in many cases the successful commercialization of a new rootstock genotype requires that license holders have the protection of a patent before they will make the large investments required to bring a new rootstock online. Also with increasing costs and decreasing public resources for breeding, the success of a breeding program is dependent on some return to the intensive investments that proper rootstock breeding requires.

10. Stage 10: First Commercial Sale of Rootstocks, Elimination of All Unreleased Genotypes from Trials, Years 28–30. Data collection continues for NC-140 and on-farm grower cooperator trials. Unreleased genotypes that showed promise but were not demonstrably superior to commercially available rootstocks are eliminated from the program or selected for release in alternative markets (ornamental, etc.).

11. Stages 1–8: Integration of Marker-Assisted Breeding. Where possible, a significant portion of the breeding project should be dedicated to improving current breeding techniques and screening methods to accelerate development of germplasm with multiple resistances to biotic and abiotic stresses. Developing knowledge on the nature and inheritance of traits is necessary to produce horticulturally superior germplasm with commercial potential. Marker-assisted selection has been shown to increase the occurrence of dwarfing and highly productive genotypes selected, and has bypassed the need for poorly replicated first test orchard trials and entering advanced testing with many replications per rootstock genotype. The MAB process has the potential to decrease the breeding period by one-third over conventional methods because it can significantly decrease the evaluation time required to characterize simply inherited traits that otherwise would necessitate 7–10 years for proper evaluation. This process can be used retroactively on material that has already entered the breeding pipeline. It is clear therefore that the process of marker development, validation, and implementation should be integrated in all development stages of the breeding program. In light of this, the next section describes some of the current methods for the integration of genomics/transcriptomics with breeding practices

such as parent selection, seedling culling, and identification of new trait components associated with higher productivity.

D. Leveraging Sequence Information and Gene Expression as a Guide for Marker Development

The publication of the genome sequence of apple (Velasco et al. 2010) and other genomic resources available through the Genome Database for Rosaceae (www.rosaceae.org) such as expressed sequence tag (EST) libraries (Han et al. 2007; Wisniewski et al. 2007; Chagne et al. 2008; Gasic et al. 2009a, b), EST-derived microsatellite markers (Wang et al. 2012), gene predictions aligned to the genome, genetic maps (Liebhard et al. 2003; Kenis and Keulemans 2005; Silfverberg-Dilworth et al. 2006; Celton et al. 2009), and the Breeder's ToolBox developed through the RosBREED effort (Iezzoni et al. 2010; Peace et al. 2010) are some of the resources available to apple rootstock breeding programs for the development and refinement of markers for MAB. Another useful tool is being developed with the apple collection of the Plant Genetic Resources Unit in Geneva, NY, where several thousand *Malus* accessions are being genotyped using next-generation sequencing technology in a technique called genotyping by sequencing or GBS (Myles et al. 2010; Elshire et al. 2011). This project has yielded thousands of markers and billions of sequences aligned with the apple genome that can be interrogated by means of a database and allow the identification of polymorphisms and haplotypes unique to interesting germplasm sources. In addition to the aforementioned resources, the availability of single-nucleotide polymorphism (SNP) arrays (Chagne et al. 2012; Khan et al. 2012a) and DNA expression arrays (Jensen et al. 2010, 2012; Khan et al. 2012b) has been leveraged for the discovery of polymorphisms and expression QTLs (eQTLs) in apple rootstock breeding populations. Expression QTLs are quantitative trait loci that utilize the differential expression of genes (measured in microarray or next-generation RNA sequencing experiments) as the trait of input for the QTL analyses (Druka et al. 2010). DNA polymorphism and its effects on the transcriptome of individuals of structured populations can be an efficient tool for the identification of candidate genes that co-locate with known QTLs (Shi et al. 2007; Cubillos et al. 2012a, 2012b). An attractive feature of eQTL mapping is the ability to identify novel genetic pathways controlling the expression of important genes and the identification of *cis*- and *trans*-acting elements (Min et al. 2008; Ingvarsson and Street 2011). The eQTL discovery in the Geneva breeding program has identified the location of expression regulatory sequences for more than 3,500 genes in an apple

rootstock breeding population. The program has acquired next-generation sequences (Illumina platform) of several founding parents of the breeding program (G.41, M.27, O.3, R.5, and Dolgo) and assisted by genome analysis software such as Geneious and CLC-Bio Genomics workbench (www.geneious.com; www.clcbio.com) has developed an internal database of aligned haplotypes for small genomic regions of interest such as genes whose expression is drastically changed (expressed or not expressed) identified in the eQTL discovery process. Robust, haplotype-specific PCR markers can be developed based on sequence polymorphisms such as insertion–deletion mutations or highly variable microsatellite sequences.

A modern apple rootstock breeding program should be able to devise and apply genomic and bioinformatic tools for marker-assisted breeding of apple rootstocks including identification of the genes underlying important traits and development of genetic maps and aligned genomic sequences of founding ancestors highlighting polymorphism and allelic diversity for harnessing and design of allele-specific markers for breeding. It should also be able to leverage transcriptome profiles of root, trunk, and leaf tissues for key apple rootstock breeding populations to identify segregating candidate genes associated with desirable rootstock traits.

The selection of technologies that are more cost effective per informative data point is important for efficient genotyping of selected breeding populations and founding parents. As costs for whole genome sequencing become more accessible, pursuing next-generation whole genome sequencing of key parents in the breeding pipeline, deriving their haplotype constitution at key QTLs, and designing PCR primers specific to the desired haplotype for marker-assisted breeding have proven to be an effective strategy in the Geneva breeding program. Critical to the success of implementation of marker technologies is the validation of the robustness and effect of the new markers by testing their predictability on an array of founding parents and segments of related phenotyped breeding populations as outlined in Fig. 8.7.

The Geneva breeding program has successfully used transcriptome profiling to identify pools of genes where their expression intensity is inherited and linked in coupling with desirable trait QTLs (Jensen et al. 2010, 2012, 2014). This concept was used successfully to refine markers linked to WAA resistance as gene expression data from a segregating population was used for further marker development identifying microarray feature APPLE0FR00068101 contained in the apple contig MDC015568.236 (6,831 bp long) that contained the best matching (BLAST) sequence. Several polymorphisms were identified when comparing parental haplotype contigs obtained by next-generation

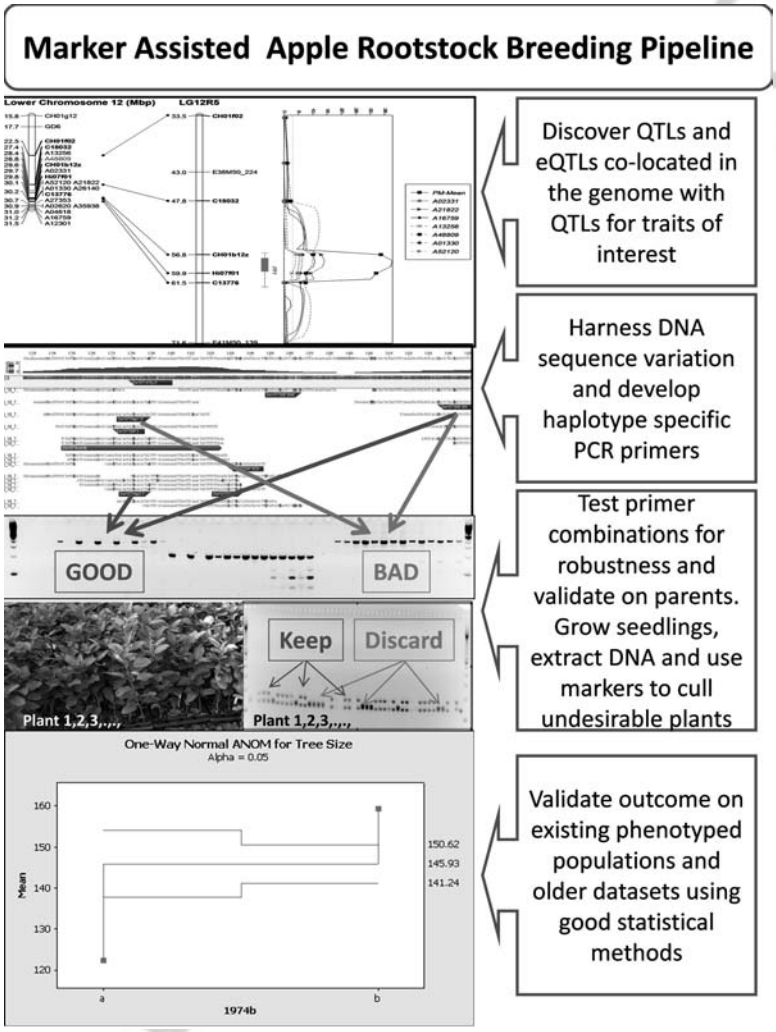

Fig. 8.7. Marker-assisted breeding pipeline adopted by the apple rootstock breeding program.

sequencing. Of particular interest for easy marker development were two microsatellite regions downstream from the BLAST hit (between base 2,500 and base 3,500) for which two sets of PCR primers were designed (Waa68101-236ssr F = GGGTTGAAGTGCGA-GAC, R = CACGCGACGAGGTATTCCAAC; Waa68101-236Indel

F = CCAAATTATGCATACAGATG, R = GATTAATGATTAGAAGAAC) and tested with parent DNA with annealing temperature gradient PCR. Both markers were polymorphic between parents but only the Waa68101-236ssr was heterozygous in the R5 parent showing bands at approximately 360, 460, and 520 bp. Segregation analysis on the O3R5 population showed very strong association of the Waa68101-236ssr 360 bp band with resistance ($P = 0.0001$) and mapped near physically linked markers in the predicted physical location of LG 17. Interestingly, in the Geneva breeding populations the Waa68101-236ssr was more predictive of WAA resistance than the published interval containing the Robusta 5-derived Er2 gene delineated between SSR markers GD96 (MDC021359.285 at 11,796 kb on chromosome 17) and GD153 (MDC013709.214 at 9,138 kb on chromosome 17) (Bus et al. 2008; Velasco et al. 2010). While no claim can be made of causality between these gene expression-derived markers and their associated traits, they do provide a good starting point for functional marker development as they are a step closer to trait expression than just random sequence-based polymorphic markers. Therefore, measuring the transcription of apple genes in several relevant tissues (root tips, phloem, and meristems) of replicated individuals belonging to structured breeding populations through direct RNA sequencing or microarray technology can aid in the discernment of a trait in progeny and refinement of the understanding of molecular mechanisms underlying that trait.

E. Value of Marker-Assisted Breeding to the Apple Rootstock Breeding Program

While theoretical benefits from the application of marker technologies to breeding have been touted in publications (Bus et al. 2000; Fazio et al. 2003; Dunemann et al. 2004), in 2011 the Geneva breeding program conducted an internal analysis of the economic impact for applying molecular markers in the breeding program by itemizing the cost per genotype for each stage of selection. The program elected to conduct the first round of MAB before stage 3, which involves the initial propagation of plants surviving Phytophthora root rot and fire blight screens. The cost of genotyping with two markers including DNA extraction and labor was about $10 per seedling. The cost to phenotype each seedling for dwarfing and precocity during stage 3 (9 years of evaluation) in 2010 dollars was $15.40 per year for 9 years = $138. The cost savings by culling non-dwarfing individuals were significant and in 2012 we were able to plant 2 orchard rows of well-replicated, high-density first test orchard instead of the 12 we had previously planted.

IV. FUTURE OF APPLE ROOTSTOCK BREEDING

While some researchers have proposed to do away with apple rootstocks and develop dwarf, columnar, own rooted, heavy bearing apple trees that produce high-quality apples, the practical and economic benefits of implementing rootstock technologies will very likely persist and continue to spur the solution of certain cultural problems by breeding new rootstocks. The starting point for any breeding is germplasm and its characterization—the notion of germplasm though will probably be increasingly about genes (different functions), specific haplotypes that promote desirable phenotypes, and possibly the use of cisgenics to transfer them from an apple accession to a rootstock (Jacobsen and Schouten 2008; Schouten et al. 2009; den Nijs et al. 2013; Jansch et al. 2014) if the technology associated with cisgenesis is exempted from regulatory burdens. Phenomics, or the thorough observation of how apple rootstocks interact with the complex artificial environment that it operates in, will play a greater role in the determination of what genotypes to select. In this context, phenomics includes the interaction with soil (pH, nutrient levels, and soil type and chemistry), with other biological elements (bacteria, oomycetes, fungi, viruses, weeds, and other roots), with the climate (temperature, precipitation, and their temporal effect), with insects (borers, aphids, and nematodes), with the scion cultivar (the effect that the scion has on rootstock development—this interaction is still very poorly understood), and finally with the human component or nursery and orchard management systems (e.g., support structures, pruning, plant growth regulators, thinning, fumigation, fertilizers, ground cover, pesticides, and herbicides). New tools that enable greater understanding of each of these interactions, such as microsensors and multispectral imaging, are becoming more available and affordable. The recent advent of instruments that monitor high-throughput screening of gene expression is enabling the observation of gene networks, the identification of which ones are associated with a desired trait or response to an interaction, and the selection of those rootstocks that increase the likelihood of expression of that trait. Among the technologies that will influence new rootstock development are (1) marker-assisted breeding and quantitative trait locus mapping, (2) gene transfer either from an unrelated source or more desirably from the same genus, (3) high-throughput DNA/RNA sequencing, (4) utilization of long-range root to shoot (and vice versa) communication through plant hormones and small and microRNAs, and (5) high-throughput affordable phenotyping of trees under different treatments.

Cooperation between scion and rootstock breeding will also become more important as new scion cultivars may require a specific set of vigor, nutrient, and hormonal parameters to yield the maximum amount of high-quality apples—even when it means that a particular rootstock gives a one apple per tree advantage over another rootstock, this advantage becomes economically noticeable with 1,500 trees to an acre. There will likely be several "designer" rootstocks that are tailored for just a few cultivars, drawing away from the one shoe (M.9) fits all idea. The propagation methods ~~of~~ such a plethora of rootstocks will likely have to change to one that can be ramped up quickly and allow a larger "portfolio" of rootstocks to be available on the market.

The future of apple rootstock breeding still has great untapped potential to impact the environment, the economy, and human nutrition in a very positive way. Given the nature of apple trees, unlocking that potential will of course need a constant and prolonged effort, which will be ever more challenging in an era of grant-funded research.

V. CONCLUSIONS

Has the process of breeding apple rootstocks changed much since the last review ~~in 1983~~ by Cummins and Aldwinckle (1983)? There are some procedures that have essentially remained the same, as one cannot select rootstocks without grafting, growing in multiple orchard settings for several years measuring growth and yield on individual trees, and then challenging the plants with the many potential diseases and stresses that affect apple rootstocks. What has changed are some of the tools and techniques available to breeding programs for understanding how apple rootstocks work (modify tree architecture) and interface with the soil physically (root penetration/anchorage), chemically (pH, CEC, nutrient extraction), and biologically (disease resistance). Another big element that has changed and that was not present in the review of 1983 is the availability of genetic, genomic, transcriptomic, proteomic, and metabolomic tools that are ever improving and the source of current knowledge about how apple rootstocks function. Another element that has increased since 1983 is the genetic base (source of new germplasm) that is available to apple rootstock breeding, as plant explorations and germplasm characterizations have resulted in the selection of new sources of disease resistance or tolerance to abiotic stresses. These advances have made possible the application of marker technologies to "assist" with breeding by improving selection efficiency—by no means a replacement for the need to evaluate trees in multiple orchards

for lengthy periods. With all the new research resources available today, it may be somewhat easier to answer difficult questions that affect apple rootstock breeding, but breeding still requires intensive investment into root research infrastructure. While ~~some~~ progress has been made since 1983, there are many questions that still need to be addressed, discoveries to be made, and improvements to be accomplished on such an ancient technology as apple rootstocks.

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