Review

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Tree Fruit and Nut Crops at the Dawn of the Pangenomic Era

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Abstract

Tree fruit and nut crops are a critical component of the global economy, producing billions of dollars of value and nourishing billions of humans every year. Improved cultivars and growing practices depend upon an understanding of the molecular basis of tree traits and physiology. Over the past 20 years, the proliferation of reference genomes for tree fruit and nut crop species has transformed the study of genetics in these crops, providing a platform for resequencing analyses of large populations, enabling comparative genomic analyses between distant plant species, and allowing the development of molecular markers for use in breeding. Limitations exist, however, with reference bias and poor transferability of markers preventing widespread applicability in many instances. As third-generation sequencing has become more accurate and accessible, a greater number of reference genomes have become available, enabling higher-quality assemblies and wider sampling of genomic diversity. To facilitate the effective use of multiple closely related genomes to create a reference and comparative genomics platform, tools for the creation of pangenome graphs have been developed, allowing for singular representations of diversity within a species or even a wider genus. Pangenomic analyses at the genus-scale have been conducted for *Malus* and *Citrus*, and more tree fruit and nut species are likely to follow. As the number of genome sequences and pangenome resources increases, the importance of generating great quantities of transcriptomic and phenomic data will increase as well. This data is essential in the drive to connect genes to traits, as is needed to develop improved tree fruit and nut crops, which can satisfy global demand.

Keywords: genomics; pangenome; transcriptomics; phenomics; tree fruit; tree nut; crop improvement; third-generation sequencing

1. Introduction

Trees, generally defined as tall (>5m) woody perennial plants with one primary stem [1], have been cultivated by humans, primarily for their nutrient-rich fruits and nuts, for at least 6000 years [2]. Today, tree fruit and nut crops contribute hundreds of billions of dollars to the global economy, providing humans with rich sources of nutrients, supplying 74% of human fruit intake [3,4]. Tree fruit and nut crops are diverse in their utility, including products which are consumed raw or minimally processed (e.g., *Pyrus communis*), used for oil production (e.g., *Olea europea*), used to produce beverages (e.g., *Coffea arabica*), and used to produce processed foods (e.g., *Theobroma cacao*). These horticultural crops and their rich array of carbohydrates, lipids, vitamins, minerals, and secondary metabolites can be preventative against metabolic diseases, cardiovascular diseases, and nutritional deficiencies [5–9].

While great strides have been made in reducing undernutrition resulting from deficiencies in caloric or protein intake, in large part due to genetic improvement of cereal crops, other forms of malnutrition remain common in many regions of the world, even as obesity rates climb globally [10,11]. As such, the world is falling short of the goal to eliminate all forms of malnutrition by 2030 (Sustainable Development Goals 2.1 and 2.2) as resolved by the United Nations member states in 2015 [4,12]. In both the developing and developed world, the accessibility and cost of a healthy, varied

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diet are at least partly to blame [13–15]. To make portions of a healthy, varied diet, such as tree fruit and nuts, available and accessible, prices for these products will need to decrease [16]. In the context of a growing global population, this necessitates an increase in supply. With most gains in agricultural productivity coming from improvements in productivity rather than increases in inputs and land usage [17], crop genetic improvement will play a vital role in creating tree fruit and nut crop cultivars that are amenable to high-density production systems. This is an opportunity for rural economic development as well, as smallholder farmers who grow horticultural crops have higher incomes and food security than other farming households [18,19]. As these issues are global in nature, genetic improvements in a wide diversity of tree fruit and nut crops will be necessary to enable this increase in production, including indigenous tree fruit and nut species, which have been historically overlooked by producers and researchers [3,20,21].

Tree fruit and nut crops come from diverse eudicot lineages; consequently, for each crop, specific genetic improvement objectives will depend on the genetic background. The domestication bottlenecks in tree fruit and nut species are relatively mild compared to many annual crops, and the "domestication syndrome", or suite of traits associated with the domestication process, appears to be different from than in annual crops [22,23]. As a result, while some newly domesticated crops, such as pecan, may retain a long juvenile period, experience alternate bearing, and have a large and unwieldy growth habit similar to their wild progenitors, most of these traits remain problems even in tree fruit and nut crops which humans have cultivated for thousands of years such as apple and European pear [24,25].

A list of common objectives for genetic improvement in tree fruit and nut species are summarized in Table 1. Prioritization of traits for improvement is highly context dependent. For example, experienced growers in the United States do not rate improvements in disease resistance as a high priority in new apple cultivars, yet it is a high priority in new cherry cultivars [26,27]. From an environmental and public health perspective, reducing pesticide applications regardless of crop species is beneficial [28], driving an interest in crops with improved disease resistance [29,30]. The wide array of stakeholder concerns that must be balanced in the process of new cultivar development contributes to diverse crop improvement objectives, with different traits suiting different use cases.

Table 1. Non-exhaustive list of common goals for genetic improvement of tree fruit and nut crops.

Trait	Goal	Example	Reference
Precocity	Developing trees that bear fruit in fewer years, and developing rootstocks that encourage early fruit bearing	Hybridization between *Juglans regia* and *Juglans hindsii* resulted in a highly precocious walnut cultivar that enters full nut production within 6 years	[31]
Salinity Stress Tolerance	Developing trees that can grow on saline soil without loss of yield	*Citrus* rootstocks, which showed enhanced production of proline and phenolic compounds, were capable of surviving on 100mM NaCl soil	[32]
Rootstock Vigor Control	Developing rootstocks that	Vigor-controlling *Malus domestica*	[33]

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	limit the vegetative growth of grafted scions	rootstocks allow for planting densities over 4000 trees/ha	
Dwarfing	Developing trees with a shorter stature to improve tree manageability and decrease unnecessary vegetative growth	*Pyrus bretschneideri* with a knockout mutation of PAT14 displayed dwarfism with shorter, thinner stems and elevated abscisic acid levels	[34]
Disease and Pest Resistance	Developing trees that require fewer pesticide applications, naturally resist existing and emerging diseases	*Carica papaya* expressing transgenic Papaya Ring Spot Virus (PRSV) coat proteins is resistant to PRSV, allowing for the recovery of the Hawaii papaya industry	[35]
Parthenocarpy	Developing trees that can produce fruit of consistent yield and quality, even in the absence of pollination; also valuable to produce seedless fruits	Tetraploid *Citrus* lines for the breeding of seedless triploid orange cultivars have been achieved via protoplast fusion	[36]
Heat and Drought Tolerance	Developing trees that can maintain yield through exceptionally high temperatures and maintain yield through exceptionally dry periods in rainfed systems	Screening for drought tolerance is essential in *Prunus dulcis*; bitter cultivars show superior qualities as drought-tolerant rootstocks	[37]
Cold Hardiness	Developing trees that can handle exceptionally low temperatures, and developing trees that do not break dormancy prematurely	Whole genome sequencing of the new cold-hardy *Malus domestica* cv. 'Hanfu' showed alterations in oligosaccharide metabolism and galactinol synthesis, which may contribute to resilience	[38]

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Self-Thinning/Decreased Fruit Set	Developing trees that will drop immature fruit in excess of their ability to develop properly	*Malus domestica* cv. 'WA 38' is self-thinning, with most fruitlets abscising following a profuse bloom	[39]
Regular Bearing	Developing trees, which produce a consistent quantity of fruit year to year	Most recommended *Carya illinoinensis* cultivars have lower than average alternate bearing indices; selection of new cultivars based on alternate bearing index is recommended	[25]
Fruit Color	Developing trees that reliably produce fruit with colors that appeal to consumer expectations	*Prunus avium* coloration is essential for the marketability of new cultivars; in response, a PCR-based assay has been developed, which can predict fruit coloration	[40]
Fruit Texture	Developing trees that bear fruit with an enjoyable eating texture, soft in some fruit and crisp in others	In the breeding of *Pyrus pyrifolia*, flesh firmness under 23 newtons was used as a selection criterion for new cultivars	[41]
Fruit Flavor	Developing fruit which have an appealing balance of sugars, acids, and secondary metabolites which contribute to aroma and other elements of taste	In *Malus domestica*, genes responsible for volatile compounds that produce apple aroma have been identified in multiple cultivars, aiding the development of new aroma profiles	[42,43]
Healthful Compounds	Developing trees with fruit that produce metabolites known to have positive effects on human health, such as unsaturated fats,	Self-pollination of the Tunisian *Olea europea* cultivar 'Chemlali Sfax' resulted in a cultivar with a greater proportion of unsaturated fats	[44]

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	antioxidants, vitamins, and minerals	relative to saturated fats	
Fruit Storage	Developing trees with fruit that can be stored long-term, processed, and transported long distances without loss in quality	Reduction of fruit browning in *Malus domestica* through the silencing of Polyphenol Oxidase via transgenic RNA silencing	[45]
Fruit Size	Developing trees that produce large fruits and nuts	A decentralized program for the breeding of *Castanea* in the United States makes nuts over 10 grams an objective for new selections	[46]
Self-Compatibility	Developing trees that can pollinate/fertilize themselves, reducing the risk of poor fruit set	Self-compatible *Pyrus pyrifolia* resulting from a gamma irradiation-induced 17Mb duplication including S-RNAse genes	[47,48]
Early Ripening	Developing trees which ripen early in the season to expand potential growing range and decreasing the risk of crop damage	Selection of early ripening *Carya illinoinensis* cultivars increases commercial viability in cooler growing regions, expanding the range of pecan cultivation	[49]
Mechanical Harvestability	Developing trees that are more suitable for automated harvesting mechanisms	Candidate genes with functional annotations including cell expansion and hormone response were found to be associated with peduncle length in *Prunus persica*, with greater length being associated with lower mechanical damage during harvest	[50]

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The long life cycles of tree fruit and nut crops, combined with the large size of individuals, create added complexity for both crop genetic improvement and the deployment of improved varieties into commercial growing systems. Most tree species have a long juvenile (immature) period during which they do not flower or bear fruit, which can last over 10 years. Consequently, the development of a new cultivar in these species can last from 10 years to over 50 years [51]. As recent examples, it took approximately 20 years to develop the apple cultivar 'Cosmic Crisp®', and longer than 40 years for the pecan cultivar 'Lakota' [52–54].

This lengthy development period and the resulting high costs make the use of genomics-assisted breeding and biotechnology efforts critical for selecting parents with desirable genetics and identifying individuals with promising traits early in the breeding program. The process of genetic marker-assisted selection, however, relies on well-characterized DNA variant-trait associations [55,56]. Quantitative trait loci-based marker-assisted selection is often unable to predict marker impacts across diverse genotypes reliably, is often poorly validated, and struggles to model complex traits. Consequently, marker-assisted selection has had a relatively small impact on plant genetic improvement [57,58]. Genomic selection, which considers a wider range of markers across the whole genome, shows greater performance in these regards [58–62]. Complex traits are often associated with variants from across the genome, even genomic regions that would not be intuitively associated with the trait of interest, largely due to the highly interconnected nature of genetic regulatory networks [63]. This, combined with the observation that genetic background influences the effects of genetic variants, has led to the proposal of the "omnigenic model", in which the whole genome is involved in the development of phenotype, and variant effects are poorly transferable between populations [63,64]. While in some rare instances, single variants may have enormous effects (e.g., total resistance to a pathogen conferred by some R genes), even traits like disease resistance tend to be controlled by many loci [65–67]. It is increasingly evident that whole-genome information is necessary to form the required trait-variant associations for crop genetic improvement.

Once a new cultivar has been developed and made available, it may still take decades for a new cultivar to accrue a significant market share. Economic risks to growers in adopting new cultivars, lag time between planting and return on investment for growers, access to capital for growers, lack of cultivar name recognition for buyers, and lack of confidence in sufficient supply of new cultivars for retailers, all inhibit the adoption of improved cultivars [51,68–71]. Due to these obstacles to the development and deployment of improved cultivars, many tree fruit and nut crops are produced primarily using old cultivars. As examples, European pear produced in the United States primarily represents cultivars developed in the 18th and 19th centuries [72], and it was not until 2018 that the 19th-century cultivar 'Red Delicious' was overtaken by 'Gala' as the most produced apple cultivar in the United States [73]. Improved cultivar adoption, however, is a social and ecological good, with benefits for agricultural sustainability and the economic development of rural areas [74–76].

Given the significant barriers to developing and adopting new tree fruit and nut cultivars, it is essential to consider integrating new technologies that can improve the efficiency of the entire endeavor. New long-read sequencing technologies, combined with high-throughput phenotyping, can accelerate the process of developing functional genomic knowledge [77,78], which can then be implemented in the improvement of fruit and nut crops. New reference genome assemblies enable identification of novel genes, identification of phenotypically relevant variants, and characterization of germplasm resources, which can enhance the selection of individuals with superior traits in breeding programs, all of which are further enabled by third-generation sequencing [79].

Mutagenesis can introduce novel genetic variation to accelerate the improvement of crops. Mutation breeding has been used for many decades to introduce mutations in a non-directed manner in a wide range of crops, including tree fruit and nut crops, though primarily in ornamental and herbaceous annual crops [80,81]. Whereas previously many novel variants arising from mutation breeding may have gone undetected, using third generation sequencing, it is more efficient to use sequencing data to identify mutations, particularly large structural variants [80,82]. Such an approach can reveal the functional genomics of structural variants [48,83], which may have difficult-to-predict

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impacts on phenotypes at present [84]. Options for site-directed mutagenesis exist now as well. The now dominant CRISPR/Cas based system was first used on a tree fruit species in *Citrus sinensis* in 2014 [85]; systems for gene editing without the integration of transgenes into the host plant now exist [86], enabling gene editing not just for the exploration of gene function but also for the creation of superior crops. These methods, however, are dependent upon *in vitro* cultivation, which, especially for tree fruit and nut species, is a time-consuming process, often requiring highly genotype-specific protocols to overcome recalcitrance [87]. Following recent development of a viral delivery mechanism for transgene-free germline gene editing in *Arabidopsis*, the application of this method to tree fruit and nut crops would be exceptionally impactful, allowing the highly limiting *in vitro* cultivation step in gene editing to be bypassed [88]. The United States, Japan, and recently the European Union have adopted regulatory standards that allow for the commercialization of cultivars produced through gene editing, which are free of transgenes [89–91], providing a promising avenue for the creation of improved cultivars.

While the regulatory burden of developing new cultivars via gene editing has decreased, this approach depends upon precise knowledge of the functional genomics of the species of interest; presently, this is a limitation to the efficient improvement of tree fruit and nut crops [92,93]. Whether crop improvement in the coming years is done by molecular-assisted breeding or by gene editing, an improved understanding of the genetics of tree fruit and nut species is necessary to produce sustainable crops that are more suitable for growers, consumers, and society at large. Fortunately, advances in the tools available in genomics, combined with other "omics" disciplines such as transcriptomics and phenomics, can accelerate the understanding of tree fruit and nut genomics to make these improved cultivars possible, whether by breeding or gene editing, and enable precision management of existing cultivars.

2. Understanding Genomes

Modern studies in characterizing plant genomes can be generally divided into two categories: reference-free and reference-guided. *De novo* genome assembly is a reference-free method that can be used to identify genes and regulatory regions, identify evolutionary processes and relationships by reference to other genomes, and serve as reference material for future experiments in the characterization of an organism's biology. Three parameters determine the quality of a genome assembly: 1) correctness, 2) completeness, and 3) contiguity [94–96]. All three characteristics of an assembly are essential: an incorrect assembly can misdirect researchers, an incomplete assembly may mislead through omission, and an assembly that has poor contiguity limits knowledge on the organization of the genome, a core component of its function. All assemblies, however, are imperfect with regard to each of these three aspects; this has led to the concept of the genomic assembly as a hypothesis about the underlying genome of the organism of interest [97]. Progressive improvements in sequencing technology, however, have allowed more of these assemblies to come closer to accurately representing the genome of the organisms they purport to describe. The first *Citrus* genomic assembly, for example, *Citrus sinensis* cv. 'Valencia' dihaploid line, had a highly fragmented reference genome (assembly N50 = 49.9 kb) generated solely using Illumina sequencing data. Now, a telomere-to-telomere phased genome is available for 'Valencia' using ultra-high sequencing depth PacBio continuous long read data [98]. Reference-free methods also exist for detecting genetic variation between samples, eliminating the need for genomic assembly, although this approach is less common [99,100].

Reference-guided genome analysis methods utilize a pre-existing reference genome to discover genetic variation in the broader pool of samples. Resequencing analysis seeks to determine the genetic variation between individuals by reference to a genomic assembly, allowing for the screening of much larger populations than is typically possible in reference-free analyses. Resequencing analysis can be used to detect single-nucleotide polymorphisms, small variants, large structural variants, copy number variation, phylogenetic relationships, and population genetic structure, all of which can provide information relevant to functional significance [101–103]. While second-

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generation/short-read sequencing may be adequate for many resequencing analyses (as opposed to reference-free methods, where advances in third-generation/long-read sequencing have made second-generation sequencing obsolete), these platforms have difficulty in the detection of large structural variants [82]. Reference-based assembly methods also exist, leveraging existing assemblies to guide the construction of a new assembly for a related organism, to enable higher-quality assemblies [104–106]. While reference-based methods are advantageous in that they enable the detection of genetic variation or novel assemblies at a lower cost, reference bias is a potential issue in such experiments. Reference bias typically favors the reference variant at a variable site on the genome [107,108], and DNA sequence reads can fail to map entirely, especially in regions of the reference genome that are specific to that individual [109]. These issues have been a driver in the increasing number of *de novo* genomic assemblies.

3. First and Second-Generation Sequencing: From Model Species to Reference Genomes

The first plant genome, that of the model species *Arabidopsis thaliana*, was sequenced using the Sanger sequencing method, with a complete assembly by 2000 [110]. The expense of Sanger sequencing for whole genome assembly generally made it prohibitively expensive to sequence organisms other than model organisms and humans, with few exceptions. A highly inbred, transgenic cultivar of papaya (*Carica papaya* cv. 'SunUp') was the first fruit species to be sequenced utilizing Sanger sequencing [111]. In the late 2000s, there was a rapid decrease in the cost of sequencing on a per-megabase basis, from about $1,000/MB to around $1, driven by the commercial release of second-generation sequencing platforms, most notably Illumina, which introduced a massively parallel approach to sequencing [112]. While Illumina platforms had shorter read lengths relative to Sanger sequencing, the quantity of data that could be produced enabled the sequencing of a wider breadth of plant species. The first temperate tree fruit species for which a reference genomic assembly was reported in 2010 was apple (*Malus domestica* cv. 'Golden Delicious') using data collected from a combination of Illumina and 454 platforms [113]. The 2010s saw a rapid increase in the number of reference genomes released for tree fruit and nut species, facilitated by these new technologies (Table 2). Reference genomes generally proliferated in accordance with the economic value of the crop. Tree fruit and nut crops of high global economic importance, such as apple [113], peach [114], and coffee [115], had published reference genomes in the early 2010s, whereas crops with more regional economic significance, such as pecan [116], chestnut [117], and guava [118] did not have reference genomes until years later. While most reference genome assemblies of this era utilize Illumina sequencing data, some notable exceptions exist, such as the *Coffea canephora* reference genome, using Sanger and 454 data [115], or the *Pyrus communis* reference genome, which was assembled solely using 454 data [119].

Table 2. First genomic reference sequence for widely cultivated tree fruit and nut genera.

Family	Genus	Species	Year	Sequencing Platform	Reference
Caricaceae	*Carica*	*papaya*	2008	Sanger	[111]
Rosaceae	*Malus*	*domestica*	2010	Illumina, 454	[113]
Malvaceae	*Theobroma*	*cacao*	2011	Illumina, 454	[120]
Rosaceae	*Prunus*	*persica*	2013	Sanger, Illumina	[121]
Rutaceae	*Citrus*	*sinensis*	2013	Illumina	[122]
Rosaceae	*Pyrus*	*bretschneideri*	2013	Illumina	[123]
Rubiaceae	*Coffea*	*canephora*	2014	Sanger, 454	[115]

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Oleaceae	*Olea*	*europaea*	2016	Illumina	[124]
Proteaeceae	*Macadamia*	*integrifolia*	2016	Illumina	[125]
Juglandaceae	*Juglans*	*regia*	2016	Illumina	[126]
Moraceae	*Ficus*	*carica*	2017	Illumina	[127]
Malvaceae	*Durio*	*zibethinus*	2017	Illumina, PacBio	[128]
Juglandaceae	*Carya*	*illinoinensis*	2019	Illumina, PacBio	[116]
Anacardiaceae	*Pistacia*	*vera*	2019	Illumina, PacBio	[129]
Ebenaceae	*Diospyros*	*oleifera*	2019	Illumina, PacBio	[130]
Fagaceae	*Castanea*	*mollissima*	2019	Illumina, 454	[117]
Moraceae	*Artocarpus*	*heterophyllus*	2019	Illumina	[131]
Rosaceae	*Eriobotrya*	*japonica*	2020	Illumina, Nanopore	[132]
Rosaceae	*Cydonia*	*oblonga*	2021	Illumina	[133]
Myrtaceae	*Psidium*	*guajava*	2021	Illumina	[134]
Betulaceae	*Corylus*	*mandshurica*	2021	Illumina, Nanopore	[135]
Anacardiaceae	*Anacardium*	*occidentale*	2022	Illumina, Nanopore	[136]

Although these reference genomes often had poor contiguity due to their inability to resolve long repetitive regions, many genomic features of interest could still be studied. Evolutionary history and phylogenetics could be assessed with greater resolution, SNVs and small structural variants could be reliably detected, gene duplications and gene loss could be evaluated, and protein-coding gene sequences could be identified in full [137]. These genomes also allowed for the use of reference-based genomics and transcriptomics methods on related germplasm, revealing genes and variants of interest in the genetic improvement and genetic history of diverse tree fruit and nut crops. For example, using the 2010 'Golden Delicious' assembly, multiple SNP-arrays for genotyping in apple were developed [138,139], the inheritance of 'Fuji' haplotypes in 'Fuji' descendants were surveyed [140], insertions and deletions in bud sports responsible for variations in color and fruit timing were identified [141], *S*-RNase genotypes responsible for pollen compatibility could be determined for cultivars [142,143], selective sweeps in the evolutionary history of apple could be identified [144], and SNPs related to a wide variety of traits including fruit flavor, disease resistance, and bitter pit susceptibility could be detected [145,146]. Similar analyses have followed the publication of reference genomes of other tree fruit and nut crops.

4. Third Generation Sequencing: High-Quality Reference Genomes to Pangenomes

Third-generation sequencing, also referred to as long-read sequencing due to its ability to sequence uninterrupted DNA fragments that are many times longer than those generated from Illumina sequencing (100-500 bp vs. 10,000-500,000+ bp), has not only allowed for the generation of higher-quality reference genomes but has also contributed to the creation of the first graph pangenomes in eukaryotes [147–149]. A pangenome is a representation of all the sampled genomic variation within a population and can be constructed at a species or a genus level. The sequencing platforms of two companies, Pacific Biosciences (PacBio) and Oxford Nanopore, are the primary platforms of this generation of sequencing. PacBio platforms use a sequencing-by-synthesis approach. In the continuous long read (CLR) method, sample DNA is ligated to hairpin adapters,

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and phospholinked nucleotides produce a signal as a polymerase incorporates them; the circular consensus sequencing (CCS)/"HiFi" method can sequence the same ligated molecule multiple times to create a consensus sequence with higher accuracy [150–152]. Nanopore platforms produce sequencing data by measuring electrical potential as nucleotides pass through a protein nanopore, and these electrical signatures can be used to predict a specific nucleotide [153] Sequencing continues until the molecule has fully transited the pore or becomes stuck, enabling reads over 1Mb in length. The primary advantages of Nanopore sequencing are the ability to sequence longer DNA fragments and a lower overall cost, while the primary advantage of PacBio sequencing is higher raw read quality [154,155].

In the 2010s, third-generation sequencing methods were significantly more expensive and provided lower accuracy compared to Illumina sequencing. Nanopore sequencing had a median nucleotide accuracy of ~60% (R7 flow cells) in 2015, improving to ~80-90% (R9 flow cells) in 2016. Meanwhile, PacBio CLR offered ~85-90% accuracy, and PacBio CCS offered >98% accuracy in practice [156,157]. This issue was typically overcome by combining the second and third-generation sequencing platforms, with third-generation reads being used to improve assembly contiguity and completeness of assemblies [158]. Directly correcting third-generation sequencing reads with Illumina data allowed for the use of third-generation sequencing data in resequencing experiments [159]. Continual improvements in the cost per base sequenced and raw read quality (>99% with Nanopore R10.4 flow cells, >99.9% with PacBio CCS) have led to more genomic assemblies utilizing only third-generation sequencing platforms [149]. Inclusion of Illumina data is unsupported by many high performing assembly tools for third-generation sequencing data such as hifiasm and Verkko, and hybrid assembly tools which can integrate Illumina data often have higher computational demands and lower performance [160–162]. Hi-C sequencing, a form of all-versus-all interaction profiling that allows for the determination of genome organization by chromatin crosslinking, is often used to complement standard third-generation sequencing data to improve contiguity and resolve large misassemblies [163,164]. This form of hybrid assembly is particularly common in modern genomic reference assemblies, and integration into assembly algorithms is provided in some tools, such as Hifiasm [160]. Combining Nanopore and PacBio reads is another form of hybrid assembly used in reference genome construction, which is supported by multiple assembly tools [160,165]. Hybrid assembly now often entails the combination of multiple forms of third-generation sequencing data, reflecting the maturity of the technology.

To realize the full potential of third-generation sequencing, purpose-made algorithms for the handling and assembly of noisy, long-read data have been developed; over 900 published packages have been catalogued by long-read-tools.org [166]. While improvements in DNA sequence read accuracy have been driven in part by advancements in the actual sequencing methodology, with both Nanopore and PacBio platforms improving from <90% accuracy to >99% accuracy over the past decade, algorithmic correction of raw reads has also contributed to improvements in the usability of third-generation sequencing data. Tools for the assembly of third-generation sequencing data often include error correction as a step preceding overlap calculation. Scalability is a key issue in the analysis of long-read sequencing data, especially in the genomics of eukaryotic organisms; tools that scale poorly to large genomes, such as Canu, have been increasingly replaced by faster alternatives such as Hifiasm [160,165,167]. With new error correction and assembly tools, it is now possible to create gapless genomic assemblies using Nanopore sequencing alone, even in eukaryotes with large genomes [168].

With the expanding accessibility and quality of third-generation sequencing-enabled genomics projects, these methods are now being applied to tree fruit and nut crops. As a result, species with genomes that had been assembled using second-generation sequencing data now possess far more advanced and improved genomic assemblies. Representative of improvements in sequencing technology over the past 15 years, the previously mentioned reference genome for the 'Golden Delicious' cultivar of apple [113], was assembled with greater contiguity (N50: 111.6 kb) using Illumina and PacBio data in 2016 [169], and finally assembled telomere-to-telomere, haplotype-

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resolved using a combination of PacBio CCS, PacBio Hi-C, and Nanopore data in 2024 [170]. Advancements in sequencing technology have enabled the genomic assembly of crops with lower global economic relevance, such as persimmon and cashew (Table 2).

Resequencing analysis is also aided by advances in third-generation sequencing. Structural variants (SVs), genomic variants greater than 50bp in length, make up a small number of variants in the genome, but contribute to a relatively high proportion of the variation between individuals. While short reads are not reliable for the detection of many SVs, especially complex SVs, long reads are much more capable of resolving these variants; this variation has been relatively unexplored as a consequence of sequencing technology limitations [82,171]. While most attention has been paid to the revolutionary impact of third-generation sequencing on de novo genomic assembly, its ability to improve reference-based methods also aids in the connection of genetic variants to crop plant phenotypes.

A new form of genomic construction, the pangenome, has also been enabled in eukaryotes by third-generation sequencing. Pangenomic analysis of prokaryotic organisms has been feasible for approximately 20 years due to their shorter and less repetitive genomes [172,173]. A pangenome (sometimes called a "super pangenome" when applied to the genus level) is a set of genomes that encompasses a large proportion of the diversity within a given taxon, generally a species or genus [148,174]. Whereas reference genome assemblies have typically been made for cultivars of species with high economic relevance, pangenomes, especially super pangenomes, normally include genotypes that are not under cultivation or are not major components of commercial cultivation [148]. This approach can distinguish regions of the genome that are shared among all members of the taxon, known as the core genome, from regions that are not present among all members of the taxon, referred to as the variable or dispensable genome [174]. As it has become increasingly apparent that genetic background and thus the whole genome influences the effects of alleles on traits [63,64], pangenomic analysis, based on the whole genome analysis of many individuals with diverse backgrounds, seeks to enable genomic analyses that have more predictive power than previous reference-based studies.

Pangenome analyses of tree fruit and nut crops started with the extensively cultivated *Malus* and *Citrus*, with *Malus* being represented by 30 accessions from 30 species, and *Citrus* being represented by six new assemblies from six species combined with six existing assemblies and six assemblies from *Citrus*-related genera [175,176]. These analyses allowed for taxonomic reclassification, the identification of selective sweeps, and the identification of genes essential for cultivation value, such as the role of PH4 in citric acid accumulation in *Citrus*. These analyses also produced pangenome graphs, a single graph-based assembly composed of sequence nodes connected by edges that attempt to model all genetic variants within a taxon of interest; construction of a pangenome graph is generally performed using haplotype assemblies as inputs [177]. For Eukaryotic organisms, Minigraph and Minigraph-Cactus are the most commonly used tools for pangenome graph construction, the former producing a graph of structural variants, and the latter building from a structural variation graph to create a "lossless" graph which includes small variants [177–179]. PGGB is another tool for constructing pangenome graphs at all scales. Unlike Minigraph and Minigraph-Cactus, it does not rely on a fixed reference, but it has poor scaling due to its all-vs.-all alignment stage [177,180]. As plant genera are often quite divergent and rich in structural variation, the development of computationally efficient and scalable references may be particularly useful. Pangenome graphs can provide references that can be used in resequencing studies to decrease the reference biases of isolated reference genomes and significantly improve error rates, especially aiding in the detection of rare structural variants [181–183]. Where many single genome references may omit relatively common genetic variants within a clade, leading to issues such as missing heritability in GWAS experiments, variant calling with the aid of a pangenome graph enables the detection of more variants that may be influential in traits of interest, such as fruit soluble solids [184]. One ensemble pipeline reported a F1-statistic of 0.95 using 5X coverage 150bp paired Illumina reads when using 50 member pangenome graphs in varied plant lineages [185]; pangenome-enabled

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high quality mapping even at very low coverage may enable resequencing analyses with far broader scope.

The relative ease of pangenome analysis and pangenome graph construction using third-generation sequencing opens new possibilities for rapidly gaining an understanding of the genetics of lesser-cultivated and orphan crop species, enabling the application of improvement methods such as marker-assisted breeding and gene editing, which have primarily been applied to more intensively cultivated crops [186–188]. Pangenomics also creates opportunities for the assessment and usage of wild relatives in crop improvement efforts, either by hybridization or the identification of useful variants in wild germplasm, which can be introduced via gene editing [189–191]. As the volume of genomic data rapidly increases, further integration of other "omics" data is essential to make the most significant impact for crop genetic improvement.

5. Transcriptomics: A Tool for Identifying Function and Gene-Linked Variation

Transcriptomics, the study of the sum of RNA transcripts within an organism, is complementary to genomics. Identifying genes and their expression across diverse environmental and developmental conditions is essential in making effective use of genomic data. Annotation of reference genomes with expressed regions, whether based on RNA sequencing evidence or predicted in silico, is upstream of most comparative genomic analyses [192]. RNA sequencing is also a typical method for the selection of candidate genes for traits of interest; by comparing gene expression levels under different conditions, inferences can be made about gene function at scale, which can be refined with more targeted experiments [193,194]. RNA sequencing can also be used for the accurate detection of genetic variants in coding regions [195,196], though this approach is less common.

Concurrently with the enormous advancements in DNA sequencing technology over the past 20 years, there have been significant advances in RNA sequencing. Before the advent of second-generation sequencing, studies aiming to detect quantitative differences in RNA expression generally relied on microarrays or RT-qPCR, measuring changes in expression of a relatively small proportion of transcripts in an organism [197,198]. As a means of genomic annotation, Sanger sequencing of cDNA was a typical approach. The ability to conduct massively parallel sequencing on all transcripts within an organism (first converted into cDNA) expanded the ability for researchers to identify genes responsible for specific responses and phenotypes, even with limited pre-existing hypotheses, as well as increasing the throughput of gene annotation for genomic assemblies. Although some early experiments utilized 454 pyrosequencing of cDNA for RNA-Seq in plants, including tree fruit and nut crops [199–201], the vast majority of RNA-Seq experiments have utilized Illumina sequencing, with 95.6% of RNA-Seq data in NCBI SRA being Illumina data as of June 2025. At first, a paucity of effective tools for de novo transcriptome assembly was a limiting factor in the transcriptomics of model species, particularly where no reference genome was available, but a wide variety of computational tools for assembly, quality control, and downstream analysis have developed in response to the widespread use of RNA-Seq [202,203]. Second-generation RNA-Seq has been used to explore a wide array of traits in tree fruit and nut crops, including fruit size, fruit color, fruit development, fruit abscission, water use efficiency, dormancy, secondary metabolite biosynthesis, senescence, disease resistance, and cold stress [204–211]. The ability to apply RNA-Seq to nearly any experimental condition, trait of interest, and tissue type has made it a highly flexible method for discovering genes relevant to crop genetic improvement.

Third-generation sequencing platforms, with their higher maximum read length, are capable of sequencing entire RNA transcripts in a single read, both from cDNA and native RNA molecules [212,213]. While throughput may not be as high as in Illumina sequencing, transcript identification is more sensitive to read length and quality than data quantity, indicating a valuable role for third-generation sequencing in transcriptomics, especially as read quality continues to improve [214]. Beyond the ability to determine full gene isoforms without bioinformatic analysis, these methods can be performed without PCR amplification, as in Illumina, thereby eliminating a potential source of bias [215]. RNA modifications can also be directly sequenced, which has been applied to discover

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RNA methylation as a means of resistance to fungal pathogens in *Malus* domestica [216], and for the discovery of novel gene isoforms in *Citrus* [217], as examples. Despite this, Illumina remains the default platform for RNA-Seq. Relative to chromosomes within genomes, transcripts within transcriptomes are short and have low repetitive content, enabling adequate and effective mapping and assembly even with short reads, making the low cost and high throughput of Illumina sequencing primary considerations for many RNA-Seq experiments, especially for quantitative experiments where reference transcriptomes are available [218].

Just as collections of genomic assemblies can be used for pangenomic analysis, collections of transcriptomic assemblies can be used for pantranscriptomic analysis. While this approach cannot capture the genetic diversity present in the unexpressed regions, the small size of the transcriptome and relative ease of transcriptomic assembly makes it an appealing target for such analyses, which can include more individuals at a lower cost than pangenomic analyses. The pantranscriptome concept was first applied to maize (Zea mays), sequencing the RNA of 503 highly inbred individuals [219]. Among tree fruit and nuts, only Asian Pear (Pyrus pyrifolia) has had pantranscriptomic analysis, surveying 506 individuals, identifying selection against resistance genes in the domestication process, and relationships between stone cells, fruit anthocyanins, and stress resistance [220]. As in genomics, the ability to make these comparative analyses relies upon high-quality phenotypic data for a wide variety of individuals.

6. Phenomics

One of the primary goals of genomics is to link genetic sequences to their corresponding phenotypes. Accurate predictions enable informed decisions to be made during the process of plant genetic improvement and in the management of currently existing plant genotypes [221,222]. The collection of phenotypic data in tree fruit and nut crops poses a unique challenge. While the model plant Arabidopsis thaliana is about 25cm tall, a mature pecan tree is generally taller than 20 meters. Even simple agronomic measurements, such as height and yield, may require exponentially more effort as a result. Just as spatial scales involved in tree fruit and nut phenomics pose issues, the temporal scales pose problems as well. While Arabidopsis thaliana can complete its lifecycle in 6 weeks, the juvenile period of many tree fruit and nut crops can be 10 years or longer. The difficulty in collecting adequate phenotypic data for most field-grown crops has been termed the “phenotyping bottleneck”. It is a significant limitation on the understanding of gene function in many crops [223]. While new sequencing platforms have enabled the detection of more genetic variants, including large SVs, it has been acknowledged that functional information about these variants is scarce, a major limitation in applying these newly generated forms of data. New tools for maximizing throughput, automating processes, and collecting new forms of data have been developed in the hope of closing this gap.

Tools for phenotypic measurement of plants are abundant. Many aspects of plant health and physiology can be determined by spectral measurements, thereby eliminating the need for hands-on and destructive chemical assays in many circumstances. Chlorophyll content, chlorophyll fluorescence, anthocyanin content, carotenoid content, and thermal infrared emission can all be used to measure plant health and physiological responses under an array of conditions [224–227]. More specific analyses of chlorophyll fluorescence, such as Fv/Fm, a measure of photosynthetic efficiency, can be used as broadly applicable measures of plant stress [228].

Tools for rapid non-spectral measurements have also become available. LIDAR is frequently used for the measurement of plant height and 3D structure and is sufficiently precise to be used on small plant species such as wheat [229]. Real-time measurements of plant or fruit gas exchange and physiological processes are possible with sensors for O_2, CO_2, and ethylene [230,231]. Thin, non-obtrusive sensors, which can be placed on plant surfaces, have been developed for measuring a range of plant phenotypes, including elongation, leaf water status, temperature, bioelectrical potential, and stress response [232]. Root systems can be imaged in their native state using magnetic resonance imaging (MRI) and X-ray computed tomography (CT) scanning systems [233,234].

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High-throughput assessment of plant phenotypes typically requires some degree of automation. Advancements in robotics and the increasing availability of unmanned aerial vehicles (UAVs) over the past decade have made this more widely accessible. UAVs, often equipped with multispectral cameras or LIDAR sensors, can measure large areas of cultivated plants of any size in short amounts of time, ranging from field crops to forest trees [235–237]. Ground-level autonomous phenotyping systems have also been developed [238]. While high-throughput phenotyping in the field can be an effective means of studying many traits, controlled-environment systems can enable studies into rare environmental conditions or apply phenotyping tools that are unsuited to field conditions [239,240]. Open-source plans for automated phenomics chambers have become available, making systems that can provide reproducible phenotypic measurements accessible to more researchers [241–243]. For tree fruit and nut crops, growth chambers are unfortunately too small for mature trees, but relatively young trees can still be assessed with respect to a wide variety of stress conditions, which may be difficult to replicate in the field.

In vitro systems are often not ideal for phenomics, as they do not accurately replicate ex vitro conditions. Plants in vitro are typically cultivated in carbohydrate-rich media without roots, generally under stable conditions and relatively low light. The ability to examine the phenotypes of large trees on a small spatial and temporal scale, however, makes this approach appealing where it can be applied. In *Citrus*, in vitro assessment of mutants' salt stress tolerance was found to predict ex vitro salt stress tolerance, although fine comparisons between salt-tolerant genotypes were not reliable in vitro [244,245]. Traits such as drought tolerance, salt tolerance, herbicide resistance, and pathogen resistance have been successfully tested in vitro [246–251]. While only a limited range of traits can be studied under in vitro conditions, when applicable, it can be a valuable tool for developing preliminary insights into plant phenotypic responses at a low cost and in a short amount of time.

7. Conclusion

Recent rapid improvements in DNA sequencing technologies have led to new opportunities in exploring the functional genomics of tree fruit and nut crops, especially in conjunction with advances in other "omics" fields. Third-generation sequencing has expanded the capabilities of genomics researchers, particularly in the tasks of de novo assembly of highly complete and contiguous genomes, as well as the detection of large structural variants. The pangenome, a form of genomic analysis that has become possible due to advances in third-generation sequencing, allows for highly detailed explorations of genetic diversity, including wild germplasm, which often possess traits informative on climate resilience and domestication. Together with advances in transcriptomics and phenomics, the ability to connect genotypes and phenotypes to advance knowledge of functional genomics is greater than ever before.

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