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Review Article

De novo phasing resolves haplotype sequences in complex plant genomes

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Summary

Genome phasing is a recently developed assembly method that separates heterozygous eukaryotic genomic regions and builds haplotype-resolved assemblies. Because differences between haplotypes are ignored in most published *de novo* genomes, assemblies are available as consensus genomes consisting of haplotype mixtures, thus increasing the need for genome phasing. Here, we review the operating principles and characteristics of several freely available and widely used phasing tools (TrioCanu, FALCON-Phase, and ALLHiC). An examination of downstream analyses using haplotype-resolved genome assemblies in plants indicated significant differences among haplotypes regarding chromosomal rearrangements, sequence insertions, and expression of specific alleles that contribute to the acquisition of the biological characteristics of plant species. Finally, we suggest directions to solve addressing limitations of current genome-phasing methods. This review provides insights into the current progress, limitations, and future directions of *de novo* genome phasing, which will enable researchers to easily access and utilize genome-phasing in studies involving highly heterozygous complex plant genomes.

Introduction

The construction of high-quality genome assemblies is now possible due to recent advances in sequencing technologies that enable the generation of long-read sequences (LRS), including single-molecule real-time sequencing (SMRT), and Oxford Nanopore Technologies (ONT), with chromosome-scale mate-pair reads such as high-throughput chromatin conformation capture (Hi-C). However, most genome assemblies currently available for highly heterozygous diploid or polyploid species contain crucial errors because haplotype differences were ignored during the *de novo* assembly process that generates consensus chimeric sequences without separating haplotype alleles (Church *et al.*, 2011, 2015; Korlach *et al.*, 2017). Several reports have described phasing approaches for constructing haplotype-resolved genome assemblies by distinguishing sequences of haplotype alleles inherited through maternal and paternal genetic materials (Edge *et al.*, 2017; Koren *et al.*, 2018; Kronenberg *et al.*, 2021; Patterson *et al.*, 2015; Shi *et al.*, 2019; Zhang *et al.*, 2019b). Since the launching of the international HapMap project in 2002 (International HapMap *et al.*, 2007), many genomes, particularly those of animals, have been assembled at the haplotype level and deposited in public databases (Bredemeyer *et al.*, 2021; Cao *et al.*, 2015; Mott, 2007). However, haplotype-resolved genome assembly is not commonly used in plant research due to the highly non-inbred character and complex genomic structures of plants (Kyriakidou *et al.*, 2018; Van de Peer *et al.*, 2017; Wu *et al.*, 2018).

In plants, haplotype-resolved genome assembly has been primarily implemented for diploid genomes using reference-guided or *de novo* assembly based approaches. If a reference genome is available, haplotype structural variants can be identified by aligning reads with the reference genome. Each haplotype is grouped and assembled separately based on heterozygous variant sites. The generation of haplotype assemblies using the reference-guided method demands less computational work; however, the results obtained depend on the quality and structural complexity of the reference genome. *De novo* assembly based phasing generates contigs from two or more parental haplotypes by unzipping heterozygous regions and grouping them into individual haplotypes using Hi-C reads. If short-read sequences (SRS) obtained from Illumina platform analyses of parents with LRS from SMRT or ONT of their offspring are available, accurate offspring phasing is possible by identifying sequences specific to each parent and classifying the contigs of the offspring from the maternal and paternal genomes. In addition to these approaches, gamete-cell sequencing can be used to generate raw sequences of each haplotype; although this is an ideal method for constructing haplotype-level assemblies, it has technical limitations (Iqbal *et al.*, 2020; Jia *et al.*, 2016; Shi *et al.*, 2019).

Previous studies reported plant genomes assembled as chromosome-scale haplotypes and demonstrated the importance of phasing by comparing each haplotype allele with the consensus assembly. Specifically, structural variations (SVs) between haplotype alleles such as chromosomal rearrangements and single-nucleotide polymorphisms (SNPs) that might have been disregarded during consensus genome assembly have been identified. Based on the variation in gene level, a number of presence-absence variations (PAVs), allele-specific expression (ASE) patterns, and dominant-recessive alleles that contribute to phenotype alternation also have been identified (Hasing *et al.*,

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2020; Seo *et al.*, 2016; Zhou *et al.*, 2020). Here, we review various *de novo* assembly based phasing methods, focusing primarily on the most popular and freely available pipelines: TrioCanu (Koren *et al.*, 2018), FALCON-Phase (Kronenberg *et al.*, 2021), and ALLHiC (Zhang *et al.*, 2019b). Input data, operating processes, output characteristics, and limitations are discussed to provide a guide for novice researchers engaged in plant genome phasing. Finally, we present cases that illustrate significant differences between haplotypes for genomic structures, gene repertoires, and gene expression, demonstrating the fundamental importance of genome phasing in plants.

Data preparation for *de novo* genome phasing pipelines for haplotype-resolved assembly

The basic workflow for constructing a *de novo* genome assembly for each haplotype allele generally consists of (1) sequencing read data, (2) assembly and phasing, (3) scaffolding, and (4) post-processing (Figure 1). This workflow is similar to the general *de novo* assembly process but implements an additional process to assign contigs to the appropriate haplotypes to achieve the correct genome constitution. Although a variety of *de novo* phasing tools are available, we focused on illustrating the operation and characteristics of three major and freely available tools, the TrioCanu (Koren *et al.*, 2018), FALCON-Phase (Kronenberg *et al.*, 2021), and ALLHiC (Zhang *et al.*, 2019b) pipelines.

The first step in the haplotype-resolved assembly process is preparing the read data. The three pipelines basically require LRS, such as SMRT or ONT, which are more suitable than SRS given the complex structure of the plant genome, which includes a large number of repeat or heterozygous regions (Van de Peer *et al.*, 2017). An advanced option that can be used for high-quality genome phasing is PacBio High-Fidelity (HiFi) reads, which is the latest sequencing technology and exhibits high accuracy (up to 99.8%; Wenger *et al.*, 2019). A recent study reported an improved version of the haplotype-resolved human genome using HiFi reads (Nurk *et al.*, 2020). In addition to the LRS, the SRS of each parent species are essential when using the TrioCanu pipeline, and FALCON-Phase and ALLHiC require input sequences of Hi-C reads. When preparing the raw sequences for TrioCanu, preparation of parental SRS is recommended with >30× coverage for each parent and >40× LRS coverage for the target offspring (Koren *et al.*, 2018). When using FALCON-Phase, PacBio LRS or HiFi reads are needed with >60× or >30× coverage, respectively (Kronenberg *et al.*, 2021).

Phasing process using the TrioCanu, FALCON-Phase, and ALLHiC pipelines

The LRS of a diploid target genome can be classified into maternal and paternal haplotypes based on the SRS of the target's parents using the TrioCanu module in the Canu assembler. Prior to contig assembly, TrioCanu constructs a list of *k*-mer subsequences from the two parental SRS datasets and identifies parent-specific *k*-mers as distinctive markers to infer which offspring LRS are derived from which parental haplotype. The LRS of the offspring are then assigned to the maternal and paternal haplotype groups based on parent-unique *k*-mers (Figure 2a). Finally, the offspring LRS in individual haplotype groups are assembled using Canu. Because the offspring LRS in each haplotype are provided, users

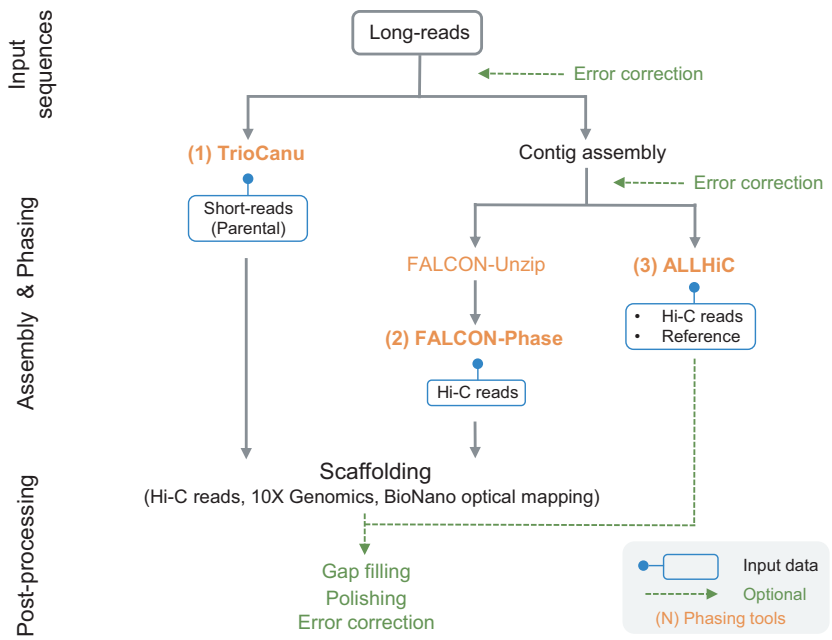

Figure 1 *De novo* genome phasing using TrioCanu, FALCON-Phase, and ALLHiC pipelines. Diagram depicts the simplified genome phasing process using TrioCanu, FALCON-Phase, and ALLHiC. The three major phasing tools are highlighted in orange. All phasing tools use long-read sequences (LRS) or assembled contigs as basic input data. Additional input data for each tool is indicated in the blue box below the tool name. TrioCanu uses LRS of the target genome and short-read sequences (SRS) of the parental genomes to construct haplotype-resolved genome assemblies. FALCON-Phase requires Hi-C reads and the results from FALCON and FALCON-Unzip. Because TrioCanu and FALCON-Phase results are generated at the contig level, scaffolding with Hi-C reads, 10× Genomics, and/or BioNano optical mapping are options for constructing chromosome-scale assemblies. ALLHiC uses assembled contigs, Hi-C reads, and genomic data of closely related species to construct haplotype-resolved genome assemblies at the chromosome level without an additional scaffolding step. Optional steps to improve the quality of haplotype-resolved genomes are indicated by green dashed lines.

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can assemble haplotype groups using other long-read assemblers such as FALCON (Chin *et al*., 2016) or Flye (Kolmogorov *et al*., 2019). Although highly accurate assemblies are possible with TrioCanu, the pipeline is difficult to use if the raw data of the parents are unavailable. To assemble without parental sequence information, FALCON-Phase and ALLHiC were developed to generate haplotype-resolved genome assemblies using Hi-C reads instead.

FALCON-Phase was designed to classify primary and associated contigs from the FALCON pipeline and generate individual haplotype assemblies for diploid genomes. FALCON basically assembles LRS into consensus sequences (referred to as primary contigs) based on a string graph regardless of haplotype variations. During graph construction, the large heterozygous variations, such as structural variants, are detected and separated into primary and associated contigs (Figure 2b). Because haplotype-fused contigs will remain as a result of ignoring small heterozygous variations such as SNPs, the FALCON module FALCON-Unzip can be used to identify haplotype-fused regions by mapping LRS to the assembly, including both primary and associated contigs. In addition, FALCON-Unzip divides mapped LRS into two groups for each haplotype-fused region, given the small heterozygous variations (Figure 2b). FALCON-Unzip then reassembles the LRS in the two groups for each haplotype-fused region and generates additional primary and associated contigs, which are fused in the same contigs (Figure 2b). Finally, updated primary contigs and haplotigs (updated associated contigs) are constructed. In the updated contigs, it is not determined which haplotype generates which primary contigs or haplotigs, and this generates switch errors because FALCON-Unzip divided the fused regions into two groups without assignments in specific haplotypes (Figure 2b). FALCON-Phase corrects these switch errors and generates two complete haplotype assemblies via Hi-C reads mapping to the updated primary contigs and haplotigs generated by FALCON-Unzip. Specifically, the assembled contigs obtained from FALCON-Unzip are separated and classified as phase blocks and collapsed regions (homozygous contigs; Figure 2b). The phase blocks are assigned to each haplotype group based on the results of Hi-C reads mapping (Figure 2b). By merging the collapsed regions (shown in grey in Figure 2b) into two sets of resolved haplotype phase blocks, FALCON-Phase generates two haplotype-resolved contig sets (Figure 2b). FALCON-Phase requires FALCON-Unzip result files, so users should assemble the LRS using FALCON and FALCON-Unzip before genome phasing using FALCON-Phase. Both TrioCanu and FALCON-Phase generate haplotype-resolved assemblies at the contig level. To construct chromosome-level genome assemblies, contigs should be ordered and oriented using additional data for scaffolding, such as 10× Genomics Linked-Reads and/or BioNano optical mapping (Figure 1).

ALLHiC is a *de novo* phasing tool that generates haplotype-resolved assemblies for complex polyploid species beyond diploid. ALLHiC was developed to enable the construction of chromosome-level assemblies of individual haplotypes using Hi-C reads without any additional scaffolding step. Unlike TrioCanu and FALCON-Phase, which utilize raw sequences of the target genome, ALLHiC requires previously assembled contigs, Hi-C reads of the target species, and protein-coding gene sequences of a close relative with their genomic positions as input data (Figure 2c). Before performing ALLHiC phasing, users prepare the input assembly that contains as many of the heterozygous contigs as possible to obtain more accurate individual assemblies, including specific sequences in individual haplotypes. Then, Canu assembler with the polyploid parameter can be one option to secure possible heterozygous contigs. ALLHiC cannot handle erratic sequences in the input assembly, such as haplotype-fused contigs; therefore, it is recommended that those erratic regions are corrected in contigs via Hi-C read mapping using juicer, juicebox, or 3D-DNA pipelines. A recent study showed that using error-corrected contigs from the 3D-DNA pipeline as input data could improve the quality of the haplotype-resolved tea plant genome (Zhang *et al*., 2021). After preparing the input data, ALLHiC first performs Hi-C reads mapping to assembled contigs and then saves linkage information for the contigs in the same and different haplotypes in BAM file format (Figure 2c). Allelic contig information is then generated, providing the names of contigs located at allelic positions by comparing the input assembly to the coding sequences of a closely related species. Based on the Hi-C mapping data in BAM files and the allelic contig information, the pruning step, which involves resolving haplotypes in the ALLHiC pipeline, is implemented to retain Hi-C linkages between the same haplotype contigs after removal of linkages between different haplotypes. Specifically, ALLHiC first removes the Hi-C linkages between allelic contigs based on the allelic contig information (Figure 2c). The Hi-C linkages between contigs in different haplotypes are primarily removed based on the Hi-C signal score (the number of read pairs per length of the contiguous contig) of the linkages to prevent the generation of haplotype-fused chromosomes (Figure 2c). The pruned contigs are partitioned into each haplotype set according to the user-defined group number (*K*), generally specified as the number of chromosomes (Figure 2c). Because contigs in the same haplotype but with low Hi-C signals are unassigned into haplotype groups, the rescue process assigns unassigned contigs to the partitioned haplotype groups based on the Hi-C signal in the original BAM file data (Figure 2c). After determining the order and orientation of the contigs partitioned in each haplotype group, a chromosome-level haplotype-resolved assembly is generated and saved in a single FASTA file (Figure 2c). If the number of haplotype sequences in the FASTA file does not match the number of chromosomes, users need to perform additional curation to correct the separation of haplotypes. For example, the haplotype-resolved genome of autotetraploid sugarcane (*Saccharum spontaneum*) was assembled into 48 super-scaffolds containing 32 chromosomes with 16 super-scaffolds after running ALLHiC (Zhang *et al*., 2018). The authors manually assigned unanchored super-scaffolds into 32 chromosomes when the specific scaffold (s) had the best Hi-C signal score with a specific chromosome (Zhang *et al*., 2018). Finally, users can determine which haplotypes are grouped into the homologous chromosomes in the FASTA file using a dot plot.

Evaluating the accuracy of the phasing tools

Previous studies generally validated individual haplotype assemblies using the three tools examined in this study via comparison to the parental genomes as a means of evaluating phasing accuracy. Koren *et al*. (2018) validated the F1 haplotypes from the two accessions of *Arabidopsis thaliana* (Col-0 and Cvi-0) via comparison with the parental data using TrioCanu. Haplotype A was covered with 99.5% of Col-0, and haplotype B was covered with 99.0% of Cvi-0. Kronenberg *et al*. (2021) also evaluated the

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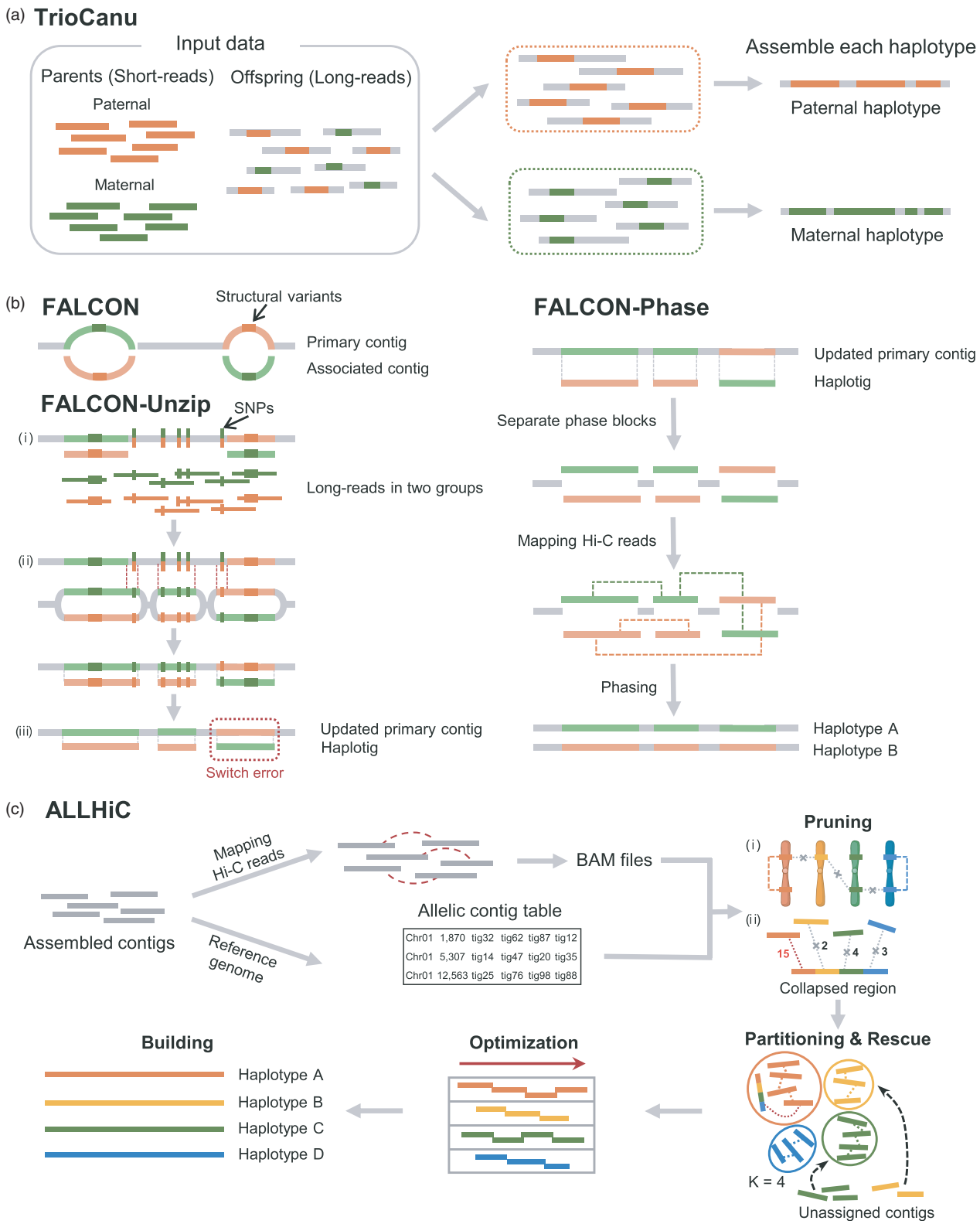

accuracy of the haplotype-resolved F1 genomes using FALCON-Phase via comparison with the parental genomes. Phasing accuracy was examined using zebra finch, cow, and human samples (HG00733 and mHomSap3). The authors reported phasing accuracies of 91%, 96%, 80%, and 91% for zebra finch, cow, HG00733, and mHomSap3, respectively. To evaluate the accuracy of phasing using ALLHiC, Zhang *et al.* (2019b) combined previously assembled *Oryza sativa* spp. *Japonica* and *O. sativa* spp. *Indica* genomes as a synthetic genome for both rice subspecies. They tested the efficiency of the pruning process and reported a reduced ratio of Hi-C linkages between different subspecies, suggesting that the pruning step improved the

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Figure 2 Detailed phasing pipelines of TrioCanu, FALCON-Phase, and ALLHiC. (a) In TrioCanu, target offspring LRS and parental SRS are used as input data. Paternal and maternal SRS are represented in orange and green, respectively. The offspring LRS are partitioned into paternal and maternal groups based on parental-specific *k*-mers. Partitioned offspring LRS in each group are assembled into each respective haplotype. (b) The FALCON-Phase pipeline proceeds according to the order FALCON, FALCON-Unzip, and FALCON-Phase. In each contig, the heterozygous regions are shown in light green and orange, and structural variants and SNPs in heterozygous regions are shown in the same dark colors. FALCON generates two types of contigs: primary and associated contigs. Associated contigs are separated from primary contigs during FALCON assembly because of structural variants between haplotypes. FALCON-Unzip consists of three steps: (i) heterozygous SNPs are detected by mapping LRS to the primary and associated contigs obtained from FALCON, (ii) heterozygous regions containing SNPs (indicated by red dashed lines) are unzipped to updated primary contigs and haplotigs, and (iii) switch error is calculated between updated primary contigs and haplotigs as depicted in the dashed red box. In FALCON-Phase, updated primary contigs and haplotigs are separated into phase blocks and a collapsed region (grey). Hi-C reads, which are marked with dashed green and orange lines, are mapped to the phase blocks. (c) ALLHiC uses assembled contigs, Hi-C reads, and the reference genome of the target species as input data. By mapping Hi-C reads to assembled contigs, linkage information between contigs can be saved as BAM files. An allelic contig table is generated using assembled contigs and the reference genome. ALLHiC resolves polyploid genomes into each haplotype via five steps (pruning, partitioning, rescue, optimization, and building). Different haplotype chromosomes and contigs are depicted in different colors. Hi-C linkages between contigs are depicted with dashed lines. Hi-C signals of Hi-C linkages are shown as numbers next to the Hi-C linkages. In the pruning step: (i) Hi-C linkages between contigs in different haplotypes are removed and (ii) collapsed regions in which some of all the haplotype regions are fused have Hi-C linkages with contigs in all haplotypes. The Hi-C linkage with the strongest Hi-C signal is marked by a red dashed line. Pruned contigs are partitioned in each haplotype group based on the *K* number (e.g., $K = 4$) in the partitioning step, and unassigned contigs are assigned to appropriate groups in the rescue step. The order and orientation of partitioned contigs are optimized in the optimization step. Finally, a haplotype-resolved genome at the chromosome level can be achieved. The figure is adapted from previous studies (Koren *et al.*, 2018; Kronenberg *et al.*, 2021; and Zhang *et al.*, 2019b) and created with BioRender.com.

phasing accuracy of ALLHiC. Based on the evaluations of several cases, these results suggest that the three tools achieve highly accurate genome phasing. However, these results are not sufficient to validate that the three tools enable accurate genome phasing for complex genomes because the evaluation cases are limited to relatively small genomes of specific species that exhibit low complexity compared with polyploid species.

Uncovering genomic information for individual haplotypes

General *de novo* genome-assembly methods are designed to generate collapsed monoploid assemblies, ignoring differences between haplotypes (Michael and VanBuren, 2020). Because the majority of published genomes were assembled ignoring differences between haplotypes, resulting in chimeric monoploid genome assemblies, these genomes could contain critical errors, such as (i) incorrect chromosome structures that result from ignoring rearrangements between individual haplotypes, especially in autopolyploid species (Tang, 2017); (ii) distorted gene repertoires resulting from the omission of genes in unassembled haplotype regions; and (iii) the presence of chimeric sequences generated by ignoring variations between haplotypes, thus hindering the identification of important haplotype characteristics such as PAV and ASE (Seo *et al.*, 2016). In this review, we examined 9 published plant haplotype-resolved *de novo* assemblies and found significant differences between haplotypes (Table 1). The difference in total length between haplotypes ranged from 1 Mb (*Medicago sativa* L.) to 130 Mb (*Camellia sinensis*; Table 1). The copy number variations of annotated genes between haplotypes were ranged from a minimum of 13 in *Vanilla planifolia* to a maximum of 6,964 in *C. sinensis* (Table 1). Genomic variations between haplotypes such as SNPs, indels, SVs, and PAVs also have been reported (Zhou *et al.*, 2020). To demonstrate the importance of constructing haplotype-resolved assemblies, we investigated and discussed significant differences between haplotypes such as chromosomal structures, haplotype-specific insertions, PAVs, and ASEs through specific examples.

Chromosomal rearrangements

Genome phasing enables the construction of precise chromosome structures for individual haplotypes, particularly for autopolyploid species. Plant genomes often undergo basic chromosome fission and fusion during evolution (Jones, 1998). These phenomena contribute to the wide range of genomic diversity observed in plants, including basal chromosome number variation and polyploidization, and serve as important mechanisms of interspecies hybridization and family ploidy speciation (Keeler and Cheplick, 1998; Svacina *et al.*, 2020). Although autopolyploid species contain three or more chromosome pairs, current assembly methods only provide single representative chromosomal structures that include haplotype chimeras but ignore rearrangements (Tang, 2017). However, haplotype-resolved assemblies could be used to distinguish chromosomal rearrangements between haplotypes in sugarcane, a complex autotetraploid species (Zhang *et al.*, 2018).

Zhang *et al.* (2018) utilized the ALLHiC pipeline to assemble the sugarcane genome using a combination of SRS and LRS phased into eight chromosome sets, each consisting of four homologous alleles. Specifically, they identified inversions among homologous alleles on chromosomes 2, 6, and 7 during two rounds of whole-genome duplication (WGD; Figure 3). Based on genomic evidence obtained by comparing individual haplotype structures, they clearly demonstrated that chromosomal inversions had occurred in the lower and upper regions of chromosomes 2 and 7 (Ss2 and Ss7), respectively, during the first WGD, which was followed by the second WGD that resulted in two inversions among a total of four haplotypes (Figure 3a). An additional inversion had occurred during the second WGD in the upper region of chromosome 6 (Ss6); consequently, one of the four haplotypes of chromosome 6 (Ss6_C) contained an inverted chromosome region (Figure 3b). These results demonstrate that genome phasing provides unprecedented genomic resources and enables the detection of comprehensive genome rearrangements in complex autotetraploid species by investigating chromosomal structures of individual haplotypes in detail.

Table 1 Summary of available haplotype-resolved plant genome assemblies

Scientific name	Ploidy	Phased in chromosome-level	Genome size (Mb)				Number of genes				Phasing pipeline	References
			HA	HB	HC	HD	HA	HB	HC	HD		
Vanilla planifolia	Diploid	O	737	744	–	–	29 167	29 180	–	–	FALCON-Phase	Hasing *et al.* (2020)
Hydrangea macrophylla	Diploid	X	2256	2227	–	–	32 205	32 222	–	–	FALCON-Phase	Nashima *et al.* (2021)
Cerasus × *yedoensis*	Diploid	X	350	340	–	–	48 280	46 796	–	–	TrioCanu	Shirasawa *et al.* (2019)
Malus domestica cv. Gala[a]	Diploid	O	658	577	–	–	46 165	40 018	–	–	DeNovo-MAGIC3	Zhang *et al.* (2019a)
Solanum tuberosum L.[a]	Diploid	O	810	800	–	–	37 115	37 094	–	–	ALLHiC	Zhou *et al.* (2020)
Saccharum spontaneum L.[a]	Tetraploid	O	734	744	723	698	21 829	21 182	20 079	20 736	ALLHiC	Zhang *et al.* (2018)
Medicago sativa L.[a]	Tetraploid	O	679	700	682	676	39 532	40 180	39 982	39 200	ALLHiC	Chen *et al.* (2020)
Camellia sinensis[a]	Diploid	O	3058	2928	–	–	29 792	22 828	–	–	ALLHiC	Zhang *et al.* (2021)
Dendrocalamus latiflorus Munro[b]	Allohexaploid	O	1374	1363	–	–	67 646	67 585	–	–	ALLHiC	Zheng *et al.* (2022)

[a]Unanchored scaffolds and genes were excluded in statistics.

[b]Statistics for allohexaploid bamboo ($A_1A_2B_1B_2C_1C_2$) were subdivided into diploid subgenomes (HA; $A_1B_1C_1$ and HB; $A_2B_2C_2$).

Haplotype-specific sequence insertions

Haplotype-specific insertions, which are a type of SV, induce sequence dissimilarities between haplotypes; these dissimilarities, in turn, affect a variety of biological characteristics, including plant phenotype (Stancu *et al.*, 2017). Because consensus genome assemblies include one of the heterozygous regions in one of the haplotypes, haplotype-specific insertions may go undetected, ultimately leading to the potential omission of genomic regions related to important traits. However, haplotype-resolved genome assemblies reflect both regions, thereby enabling the identification of trait-related genomic regions in a specific haplotype. Two examples of haplotype-specific insertion in allele-associated genomic regions that affect phenotypic alterations are described below (Figure 4).

Sun *et al.* (2020) reported the haplotype-resolved genome of the Gala variety of apple, which was assembled using DeNovoMAGIC3 (NRGene), a commercial phasing method based on Illumina SRS, 10× Genomics reads, and PacBio HiFi reads. They observed a specific long terminal repeat retrotransposon (LTR-R), denoted as redTE insertion, upstream of the *MYB* gene in haplotype B of chromosome 9, which is related to color determination in fruit (Figure 4a). This was consistent with the results of a previous study reporting that the yellowish-red color of Gala apples derives from the insertion of a single redTE LTR-R on a single side of the haplotype upstream of the *MYB* gene (Mdg_09g022880), which has a crucial role in determining red skin color in fruit (Zhang *et al.*, 2019a). They found that redTE was present only in *Malus sieversii* and *Malus domestica*, suggesting that the haplotype B-specific redTE insertion region originated from the *M. sieversii* haplotype, as demonstrated by comparing the wild-type progenitor (*M. sieversii* and *Malus sylvestris*) haplotypes and the Gala apple haplotypes (Sun *et al.*, 2020). These results demonstrate that haplotype-resolved assembly can provide accurate information regarding variations between alleles that have vital roles in the emergence of functional genes related to important agricultural traits.

The sequence diversity between dominant and recessive genes associated with growth vigour in potatoes has been characterized (Zhou *et al.*, 2020). Diploid potato (RH89-039-16) was sequenced using a combination of strategies, including Illumina whole-genome sequencing, 10× Genomics linked-reads, ONT, and circular consensus sequencing (CCS), and haplotypes were determined using the ALLHiC pipeline. The authors identified a 57-bp nucleotide insertion in *pa1* (RHC01H2G0765.2, recessive allele) that resulted in the inclusion of 19 extra amino acids in the protein encoded by the recessive allele compared with *PA1* (RHC01H1G0699.2, dominant allele; Figure 4a). This result was obtained via haplotype-resolved assembly of the potato genome and provided accurate information regarding the PAV of specific sequences in a trait-related gene.

Allele-specific expression

Not all alleles are expressed, even for the same allele in each haplotype (Springer and Stupar, 2007). Thus, genes that are actually expressed in specific haplotype(s) may not be identified in the consensus genome assembly containing a bisected gene annotation. However, haplotype-resolved genome assembly enables the entire gene repertoire of an individual haplotype to be determined, thereby enhancing our understanding of accurate allele expression.

The *CPLP* gene in the vanilla genome is an intuitive example to verify ASE in a specific haplotype. Hasing *et al.* (2020) reported a draft haplotype-resolved vanilla genome assembled via ONT LRS and Illumina SRS and phased via FALCON-Phase. They found that *CPLP* alleles (vanillin biosynthesis-related alleles) were

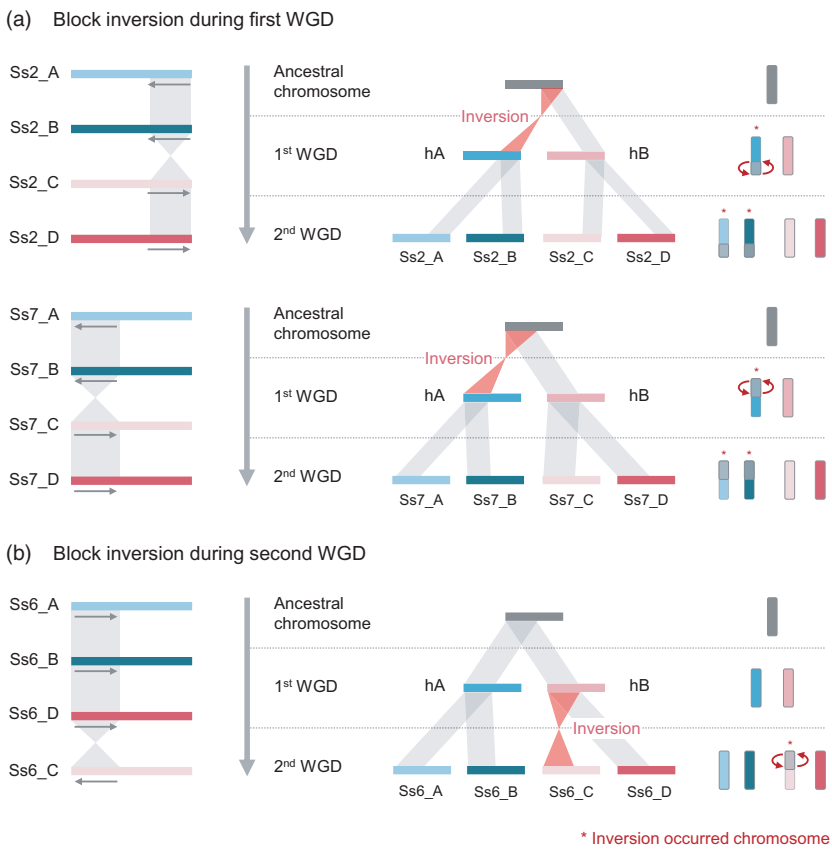

Figure 3 Chromosome rearrangements between sugarcane haplotypes. (a, b) Colored bars represent each of four haplotype chromosomes of sugarcane (Ss: *Saccharum spontaneum*). Chromosome numbers and haplotypes are presented next to Ss and underlined, respectively. Left and middle alignments show the synteny block between the four haplotypes and the predicted ancestral chromosomes. The arrow on the left under the haplotype bars indicates the direction of the alignment. The grey-shaded area shows the synteny region, and the red-shaded area indicates the inverted region. (a) Scheme of chromosomal inversion during the first whole-genome duplication (WGD) of sugarcane. The diagram shows that the long arm (short arm) in the ancestral haplotype for Ss2_A-B (Ss7_A-B) was inverted during the first WGD; the subsequent second WGD caused the duplication of the ancestral haplotypes hA to Ss2_A-B (Ss7_A-B) and hB to Ss2_C-D (Ss7_C-D), resulting in chromosome rearrangement among the haplotypes. (b) Chromosomal inversion during the second WGD of sugarcane. The Ss6 haplotypes on the left side show the reverse synteny in the short arm between Ss6_A-B-D and Ss6_C. The diagram on the right side depicts chromosome inversion specifically occurring in Ss6_C during the second WGD. The figure is adapted from a previous study (Zhang *et al.*, 2018).

differentially expressed in each haplotype (Figure 4b). Vpl_s027Ag26221, a candidate *CPLP* allele in haplotype A, exhibited relatively low transcript abundance compared with candidate *CPLP* alleles Vpl_s027Bg25947 and Vpl_s027Bg25938 in haplotype B. Vpl_s027Bg25947 was highly expressed under all conditions examined, whereas Vpl_s027Bg25938 was highly expressed in seed tissues 5–6 months after pollination. These results illustrate the contribution of ASE to vanillin biosynthesis in haplotype B. Another example of ASE is the gene encoding alcohol acyltransferase in Gala apple (*AAT1*) (Sun *et al.*, 2020). Although the sequences of both *AAT1* alleles (control ester production in Gala apple) are similar, the haplotype A *AAT1* (derived from *M. sylvestris*) is expressed at a higher level than haplotype B *AAT1* (derived from *M. sieversii*; Figure 4b). The ASE of *AAT1* alleles was due to the insertion of a specific sequence in the upstream region of haplotype A *AAT1* derived from *M. sylvestris*. These examples highlight the importance of understanding ASE as it relates to trait regulation and using haplotype-resolved assembly to accurately estimate the expression levels of each allele in individual haplotypes.

A comprehensive genome-editing protocol for complex plant genomes

The speed of genome editing can be increased using haplotype-resolved assembly based on precise genome sequence information for individual haplotypes, and this is particularly useful for polyploid species with a complex genome structure (Chen *et al.*, 2020). For accurate CRISPR/Cas9-based genome editing, identifying a specific genome target site is an essential prerequisite. However, because polyploid or highly heterozygous diploid plants generally harbor numerous dissimilar alleles, the target region may not cover all these alleles, which can ultimately decrease the accuracy of editing when target sequences are determined using only a consensus reference genome. Therefore, Chen *et al.* (2020) suggested that target regions in dissimilar alleles should be identified using the haplotype-resolved assembly of autotetraploid alfalfa (*Medicago sativa* L.; Figure 5).

The cultivated alfalfa genome was assembled using PacBio CCS LRS and Illumina HiSeq2000 SRS, and constructed as a haplotype-level assembly using ALLHiC (Chen *et al.*, 2020). The authors

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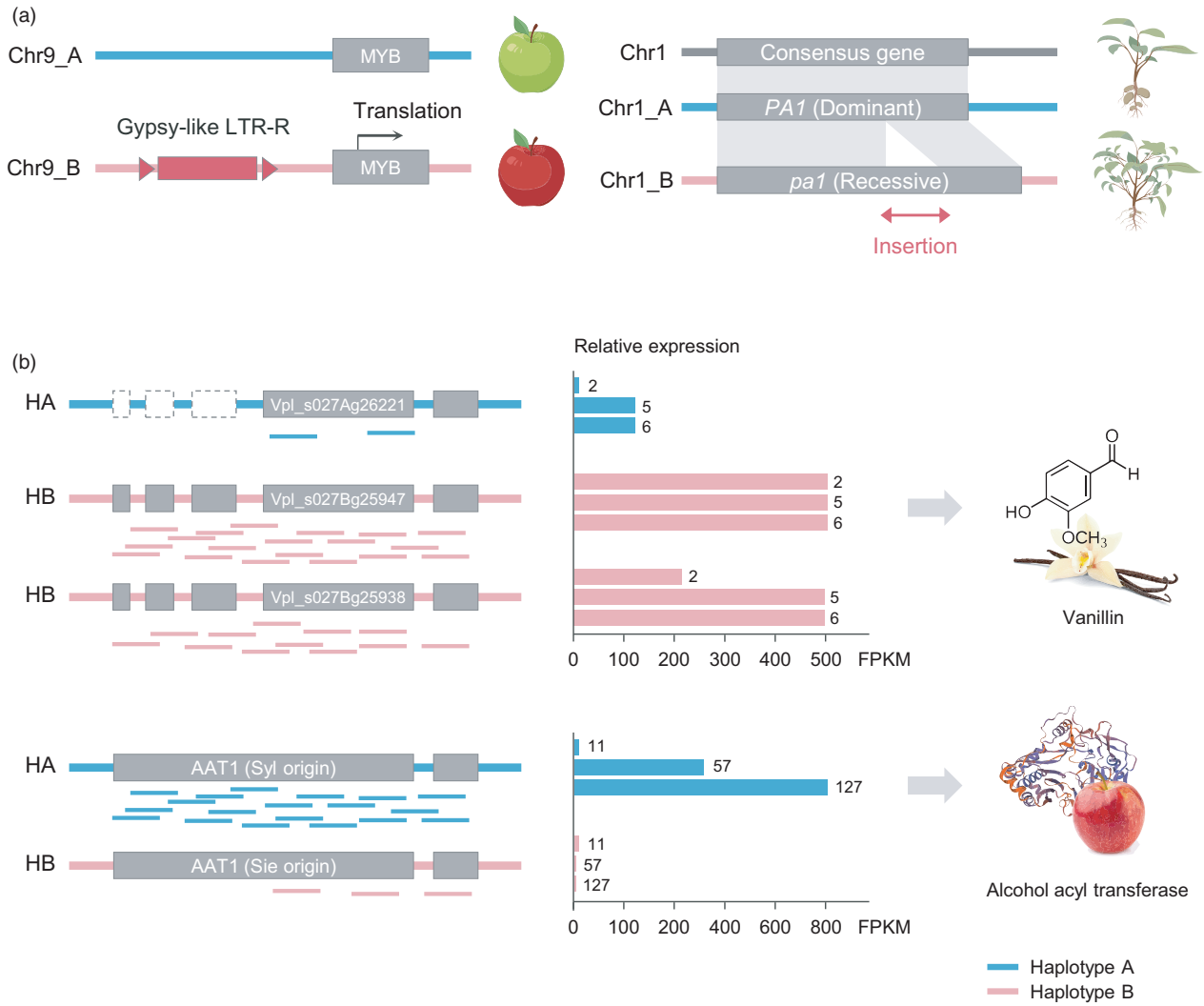

Figure 4 Haplotype-specific sequence insertion and allele-specific expression. (a) Schematic diagram illustrating phenotypic changes mediated by haplotype-specific sequence insertion. The left scheme shows that the Gypsy-like long terminal repeat retrotransposon (LTR-R) is inserted upstream of the *MYB* gene in haplotype B of chromosome 9 in the Gala apple genome. The recessive allele *pa1* (57-bp long) in potato resulted in the insertion of a sequence encoding an additional 19 amino acids compared with the dominant allele *PA1* (right). (b) Examples of allele-specific expression between haplotypes. The scheme on the left side shows differentially mapped RNA-seq data between haplotypes. Grey boxes represent exons, and dotted boxes indicate the absence of exons in the corresponding allelic position. In graphs on the right side, the numbers at the end of each relative expression bar indicate seed tissue 2, 5, and 6 months after pollination in vanilla (top) and Gala fruit (bottom) 11, 57, and 127 days after full bloom, respectively. The figure is adapted from previous studies (Sun *et al.*, 2020; Hasing *et al.*, 2020; and Zhou *et al.*, 2020) and created with BioRender.com.

established an efficient CRISPR/Cas9-based genome-editing protocol to accurately identify target regions covering four dissimilar alleles using the haplotype-resolved genome assembly of alfalfa. Allele-aware optimal guide sequences are designed as follows using this protocol (Figure 5): (i) homologous alleles of a target gene are screened based on alignments among allele sequences in each haplotype; (ii) multiple guide sequence candidates are extracted from these homologous alleles using Perl scripts (https://github.com/stanleyouth/-/blob/master/crispr.sgRNA.
finder.pl); and (iii) optimal guide sequence(s) are selected from among the multiple guide candidates based on specific evaluation criteria, such as covering all alleles, clear off-target sites, guide sequence position, and conservation. Chen *et al.* (2020) verified the feasibility of this protocol for a clearly mutated gene in the alfalfa genome, *MsPDS*, which mediates the formation of pentafoliate leaves in legume plants (Chen *et al.*, 2010). These results suggest that allele-aware genome editing is an accurate and rapid alternative solution for molecular breeding of complex polyploid or highly heterozygous diploid plant genomes to speed up the breeding procedure by exactly targeting all alleles in the haplotype.

Conclusions and perspectives

Current advanced sequencing technologies generate considerably longer sequences and chromosome-scale mate-pairs than was possible with previously described methods. Current *de novo* assembly methods are designed to generate consensus monoploid assemblies by integrating genomic sequences without considering differences between haplotypes. This results in the

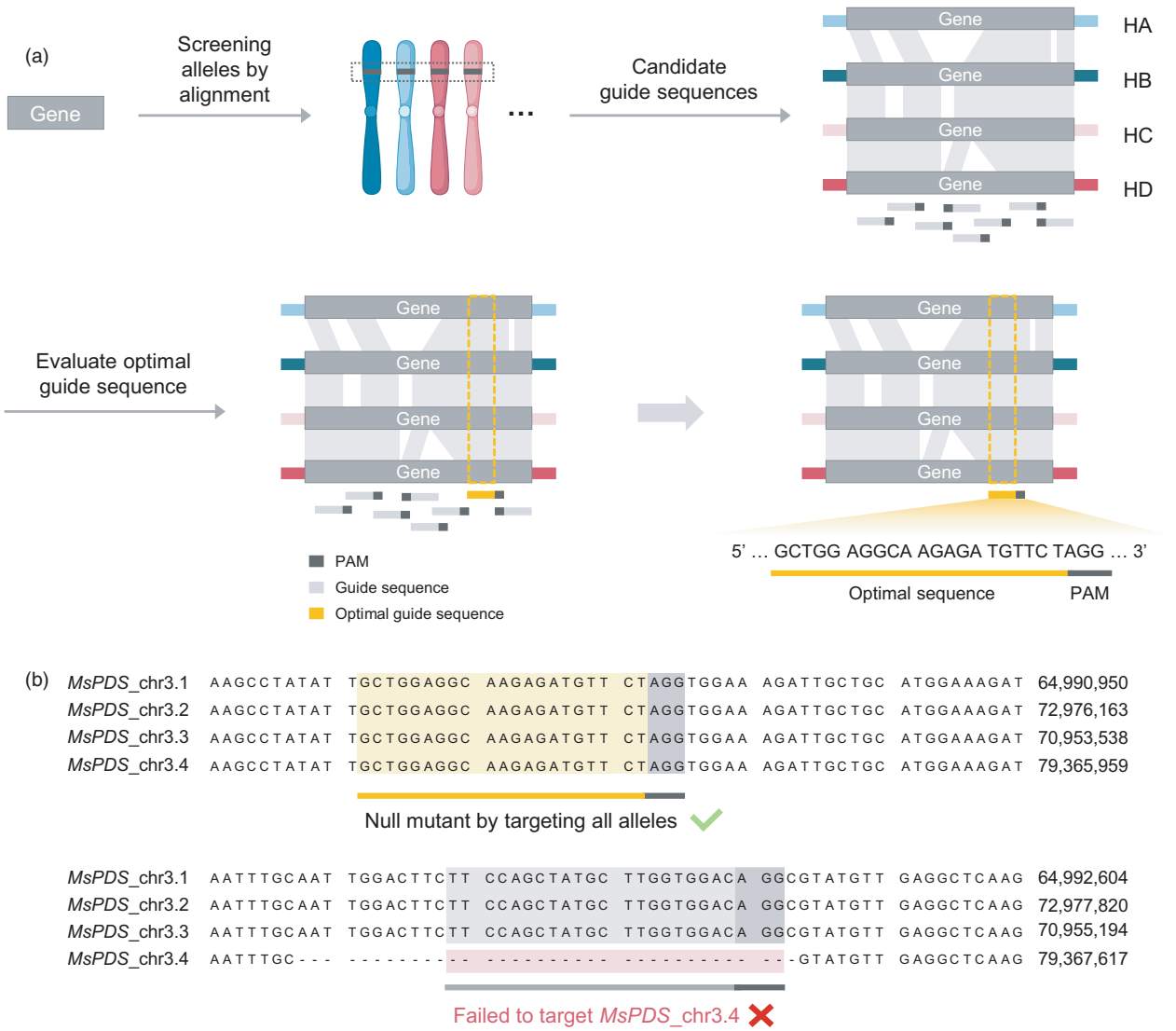

Figure 5 Genome-editing protocol using haplotype-resolved assembly of autotetraploid *Medicago sativa* L. (a) Protocol for obtaining optimal guide sequences for CRISPR/Cas9. Chromosome colors represent different haplotypes: HA, HB, HC, and HD. Dark-grey bars on each haplotype indicate alleles. Grey-shaded areas show corresponding blocks between alleles. (b) Validation of the optimal guide sequence targeting for all alleles. The top alignment is an optimal case in which sequences contain all alleles, and the bottom alignment is a suboptimal case in which sequences contain unmatched regions. Sequences following the suboptimal candidate are artificially generated for intuitive understanding. Numbers to the right of the sequence represent the chromosomal position. The figure is adapted from a previous study (Chen *et al.*, 2020) and created with BioRender.com.

generation of incorrect assemblies, especially for complex plant genomes. Genome phasing is a recently proposed alternative approach for constructing haplotype-resolved assemblies for highly heterozygous genomes.

Our investigation revealed that the representative freely available and widely used phasing tools TrioCanu, FALCON-Phase, and ALLHiC are suitable for assembling genomes of individual haplotypes, even in complex autopolyploid plant species beyond diploid plants. TrioCanu and FALCON-Phase are designed for haplotype-resolved assemblies only in diploid genomes. In TrioCanu, haplotype information from parental sequence data makes it possible to partition reads into haplotypes with high accuracy. FALCON-Phase is developed to create a haplotype-resolved genome by utilizing information on large SVs detected in FALCON and heterozygous SNPs detected in FALCON-Unzip without parental data. Unlike the above two tools, ALLHiC is specially designed for polyploid genome phasing, which can be useful in phasing polyploid plant genomes. The results of downstream analyses using haplotype-resolved assemblies indicated the importance of haplotype-resolved assemblies in plants as tools for enhancing our understanding of the fundamental characteristics of species by identifying crucial variations among haplotypes. The results of haplotype sequence-based genome-editing studies suggest that haplotype-resolved assemblies that generate optimal target sequences might be valuable tools for efficient breeding research for complex autopolyploid species.

Despite the necessity of haplotype-resolved assemblies in plant research and breeding, there are two major limitations to consider for future studies: (i) the dependency of these tools in the phasing process and (ii) the lack of standard validation methods of phasing accuracy. Because TrioCanu relies on raw

Table 2 Estimation of switch errors in published haplotype-resolved genome assemblies using the calc_switchErr pipeline

		Generated results in this study			
Scientific name	Phasing pipeline	Total number of SNPs	Total number of switched SNPs	Percentage of switch error (%)	Percentage of switch error in other studies (%)
Vanilla planifolia	FALCON-Phase	565 976	248 779	44.0	NA
Malus domestica cv. Gala	DeNovoMAGIC3	639 051	141 656	22.2	29.6 (Zheng *et al.*, 2022)
Solanum tuberosum L.	ALLHiC	209 522	35 823	17.1	15.4 (Zheng *et al.*, 2022)
Camellia sinensis	ALLHiC	371 067	27 775	7.5	5.9 (Zhang *et al.*, 2021)

The evaluation of switch errors was only performed when both raw short- and long-read sequencing data were available and assembly phased at the chromosome level.

10× Genomics-linked raw reads data were excluded from input data during evaluation for the criteria consistency between the various phasing pipelines.

sequences of the parental species as input data for accurate phasing, the tool is difficult to use in cases where parental materials are not available. Unlike TrioCanu and ALLHiC, which can use any contig assemblers, FALCON-Phase is compatible only with contig assemblies from FALCON, although more suitable tools can be used for contig assembly (Murigneux *et al.*, 2020). ALLHiC overcomes the obstacle of TrioCanu and FALCON-Phase, which can only be applied to the diploid genome; however, its application is restricted because of the requirement for whole-gene information of closely related species. These dependency problems suggest a future direction for a phasing tool update based on the classification of parental-specific haplotypes only using offspring sequences and the operation of phasing tools with any assemblers and without genomic information of closely related species.

The accuracy and quality of haplotype-resolved assemblies were assessed as described above (the 'Evaluating the accuracy of the phasing tools' section). However, it is difficult to generalize and conclusively state that those phasing tools enable the construction of accurate haplotype-resolved assemblies in most cases. Therefore, it is necessary to develop more robust approaches to estimate the accuracy level of haplotype-resolved assemblies, suggesting the need for a qualified standard of phasing accuracy. A recent evaluation of phasing accuracy offers an alternative solution. Zhang *et al.* (2021) used a new tool called calc_switchErr to assess the phasing accuracy of the tea plant (*C. sinensis*) genome, which was resolved into haplotypes using ALLHiC. The calc_switchErr tool was designed to identify the oppositely located SNPs (designated as switched SNPs) and common SNPs (designated as truly phased SNPs). For example, if haplotype A and B after genome phasing have C and T in a specific site, but calc_switchErr detects T and C in the same position of haplotype A and B, respectively, this switched SNP is classified as an error. The calc_switchErr tool estimates the error rate by calculating the ratio of switched SNPs to truly phased SNPs [(the number of switched SNPs) × 100 ÷ (the total number of truly phased SNPs)]. The accuracy of the haplotype-resolved tea plant genome was evaluated with a switch error rate of 5.9% (8473 of 144 868) which was much lower than the switch error rate of the monoploid genome (23.6%, 94 273 of 399 821) (Zhang *et al.*, 2021). To estimate the phasing accuracy using the method for haplotype-resolved plant genomes reviewed in this study, we used calc_switchErr to validate four plant genomes with raw sequences available in public databases (Table 2). Our results were slightly different from the results in a recent study due to differences in the input data used by the two studies (Zhang *et al.*, 2021; Zheng *et al.*, 2022). For example, we were unable to use 10× Genomics reads data, which were not available in public databases (Table 2). As a result, our data revealed specific scores for phasing errors of haplotype-resolved assemblies, providing standards for researchers to evaluate the phasing quality of those assemblies. The calc_switchErr tool is open-source and, thus, applicable in most cases to estimate the accuracy of haplotype-resolved assemblies by calculating the error score.

Our review provides new insights into the details of phasing principles to enhance the practical understanding of genomics researchers. We also provide valuable information regarding biological trait-related sequences in haplotype-resolved assemblies in plants. Research in plant genome phasing and the development of phasing pipelines are still in the early stages. We suggest that genome phasing will become indispensable for understanding biological phenomena and accelerating breeding research for complex plant species by overcoming current limitations.

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Conflicts of interest

All authors declare they have no conflicts of interest.

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Author contributions

S.K. designed and conceptualized the review. J.-Y.G., Y.M.L, and S.K. reviewed phasing operation principles, and M.-J.J., J.-W.C., and S.K. reviewed examples of downstream phasing analyses. J.-Y.G and M.-J.J wrote the initial manuscript, and all authors edited and reviewed the final version.

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