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REVIEW

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# Advances in gene cloning and functional genomics approaches for wheat (*Triticum aestivum* L.) improvement

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## Abstract

Wheat (*Triticum aestivum* L.) is a globally important cereal crop which provides ~20% calories in human diet. Identifying genes and elucidating their functions is still challenging in wheat due to its large genome (~16 Gb) with significant amount of repetitive elements. However, the gene cloning and functional characterization efforts are gradually picking up over the last decade. In this review article, we discussed the recent advances in wheat functional genomics including strategies (beyond the map-based cloning) based on next-generation sequencing, association analysis, and bulk segregant analysis along with its modifications. We also discussed how functional genomics approaches including conventional, genetic engineering, genome editing, and transient gene expression can be utilized in wheat improvement. Besides recent developments, we discussed the possible opportunities available for improving the field of gene cloning and functional genomics in wheat. Introducing the innovative and advanced functional genomics tools helps in identification and functional validation of target genes in wheat for faster genetic gain.

## Plain Language Summary

Wheat is an important cereal crop supplying food for ~30%–40% of the global population. Therefore, genetic improvement of wheat is important to ensure global food security. The large and complex wheat genome hinders the identification and validation of genes associated with key agronomic and stress related traits. However, recent advances in next-generation sequencing and gene editing technologies have made it easier to identify and study gene functions. In this article, we discuss the latest functional genomics tools and techniques, as well as future opportunities for advancing gene cloning and functional genomics in wheat.

**Abbreviations:** BSA, bulk segregant analysis; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins; GBS, genotyping-by-sequencing; GWAS, genome-wide association study; IWGSC, international wheat genome sequencing consortium; NGS, next-generation sequencing; NLR, nucleotide-binding leucine-rich repeats; RNAi, RNA interference; VIGS, virus-induced gene silencing; WGS, whole-genome sequencing.

Santosh Gudi and Khizar Razzaq contributed equally to this work.

## 1 | INTRODUCTION

Wheat (*Triticum aestivum* L.) is a globally important cereal crop with annual production of ~770 million metric ton (https://www.world-grain.com) and serves as a major dietary source for around 35% of the world's population (Gudi, et al., 2022a). It originated about 8500–9000 years ago in the Fertile Crescent in the Middle East by the hybridization between a cultivated tetraploid wheat (*Triticum turgidum* L., AABB) and a diploid wild progenitor species, *Aegilops tauschii* (D-genome donor) (Dubcovsky & Dvorak, 2007). Nutritional significance coupled with genomic plasticity and broader adaptability have contributed to the early domestication and widespread cultivation of wheat across the diverse agroecological regions. During the domestication and post-domestication processes, wheat has undergone extensive diversification through natural and artificial selections, which resulted in the development of several landraces and cultivated varieties with greater adaptability to various geographical regions and climatic conditions (Maccaferri et al., 2019).

Despite the wider adaptability and nutritional significance, the large (~16 GB) and complex genome limits the application of various genetic and functional genomics techniques in wheat, which have otherwise proven to be very effective in other crop species (Appels et al., 2018; Brenchley et al., 2012; Singh et al., 2022). To overcome these challenges, the International Wheat Genome Sequencing Consortium (IWGSC) has released the fully annotated, high-quality wheat reference genome "IWGSC RefSeq v1.0" (Appels et al., 2018) followed by "IWGSC RefSeq v2.1" (Zhu et al., 2021) with improved genome assembly and gene annotations. Recent advancement in next-generation sequencing (NGS) also facilitated the whole-genome sequencing (WGS), whole-genome re-sequencing, and reduced representation (RR) sequencing of wheat genomes to generate huge genomic data (Athiyannan et al., 2022; Bennett et al., 2023; Kale et al., 2022; Sato et al., 2021). Additionally, the development of high-throughput genotyping technologies, such as genotyping-by-sequencing (GBS) (Elshire et al., 2011), single-nucleotide polymorphism (SNP) arrays (Sun et al., 2020), and skim sequencing (Van Tassell et al., 2008) has enabled the genotyping of thousands of wheat germplasm lines. These tools facilitated genome-wide association studies (GWASs) (Gudi et al., 2023, 2025a, 2025b, 2025c, 2025d; X. Liu et al., 2025; Tian et al., 2023), bulk segregant analysis (BSA) (Michelmore et al., 1991), and genomic selection (Meuwissen et al., 2001) for various traits in wheat. Furthermore, the advancements in computational and bioinformatics tools facilitated the analyses of large genomic data generated by high-throughput sequencing and thereby helped identifying candidate genomic regions associated with specific traits of interest (Mészáros et al., 2023).

**Core Ideas**

- Advances in genomics and functional genomics tools, such as next-generation sequencing (NGS), helps in accelerating genetic improvement of wheat.
- Functional genomics tools help in identification and characterization of genes associated with key agronomic traits.
- Cutting-edge genome editing techniques, including gene knockout and gene knock-in, offer powerful approaches for functional validation of targeted genes.

Once such candidate genomic regions are identified, they can be utilized to develop gene-based molecular markers to facilitate marker-assisted selection (Gudi et al., 2020; Ribaut & Hoisington, 1998), marker-assisted backcrossing (Randhawa et al., 2019), and marker-assisted recurrent selection (Bernardo & Charcosset, 2006) to accelerate wheat breeding programs. Additionally, candidate genes identified from the available genomic information needs to be validated using various reverse genetic tools such as mutagenesis, transgenesis, RNA interference (RNAi) (Hannon, 2002), and genome editing techniques such as zinc-finger nucleases (ZFNs) (Porteus & Carroll, 2005), clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins (CRISPR-Cas9) (Doudna & Charpentier, 2014), and transcription activator-like effector nucleases (TALENs) (Joung & Sander, 2013). The CRISPR-Cas9 technology has revolutionized the functional genomics research by precisely editing targeted genes, which helps in creating novel allelic variants not existed in nature.

Even with these advancements, the vast majority of genes in the wheat genome have not been fully characterized or assigned specific functions. This is owing to the intricate nature of the wheat genome and the recalcitration of wheat to functional genomics analysis. Therefore, it is important to improve the available techniques and introduce innovative and advanced functional genomics tools in the wheat functional genomics toolbox to facilitate easy identification of gene functions and their regulatory networks. In this review, we have discussed the advances in genomics tools aimed at understanding the complexities of wheat genome, functional genomics tools, and the latest gene cloning/identification techniques beyond the conventional map-based cloning strategies. In the later sections, we have discussed the future opportunities in wheat functional genomics based on the recent advancements in other crop and model plant systems.

## 2 | CLASSICAL GENE IDENTIFICATION TECHNIQUES: MAP-BASED CLONING

Map-based cloning also referred as positional cloning is a fundamental molecular biology process to identify the specific location and function of a gene responsible for target traits (Table 1). The basic requirements for the map-based cloning involves (i) identification of phenotypically contrasting germplasm lines; (ii) establishing mapping populations such as $F_2$, backcross generations, doubled haploids (DHs), recombinant inbred lines (RILs), and backcross inbred lines; (iii) linkage map construction and quantitative trait loci (QTLs) mapping to identify the significant marker-trait associations; (iv) fine mapping with additional markers and larger segregating populations; and (v) functional validation of identified gene through reverse genetic approaches (viz. creating transgenics or null mutants) (Hatta et al., 2019). This approach is highly reliable as it is based on Mendelian genetics and enables precise identification of causal genes through recombination mapping with minimal or no false negatives. It has been successfully employed to clone several important wheat star genes, including genes governing vernalization (*VRN1*, *VRN2*, *VRN3*, and *VRN4*) (Kippes et al., 2015; Yan et al., 2003, 2004, 2006), high grain protein content (*Gpc-B1*) (Distelfeld & Fahima, 2007), amylose content (*SsI-D1* and *SSIVb*) (Leterrier et al., 2008; Z. Li et al., 1999), powdery mildew resistance (*PmCH1357* and *Pm41*) (F. Chen et al., 2019; M. M. Li et al., 2020a), leaf rust resistance (*Lr10*, *Lr21*, *Lr34*, and *Lr67*) (Feuillet et al., 2003; Huang et al., 2003; Krattinger et al., 2009; Moore et al., 2015), stripe rust resistance (*Yr36* and *Yr15*) (Fu et al., 2009; Klymiuk et al., 2018), stem rust resistance (*Sr8155B1*) (Shen et al., 2025), *Fusarium* head blight (FHB) resistance (*FHB1* and *WAK2*) (Gadaleta et al., 2019; S. Liu et al., 2008), spike architecture and grain yield (*TaCol-B5*) (X. Zhang et al., 2022), leaf width (*TaWAK2-TaNAL1-TaDST*) (D. Du et al., 2024a), and multiovary-producing florets (WUSCHEL-D1) (Schoen et al., 2025), in wheat.

Despite notable achievements, the widespread application of positional cloning is limited in wheat due to several challenges. For instance, constructing mapping populations is a time-consuming process because of longer generation cycle of approximately 155–160 days per generation (Gudi, et al., 2022). Additionally, QTLs identified through conventional linkage mapping studies have larger physical distances per unit of genetic distance (Varshney et al., 2014). Identifying causal genes from such QTLs is not an easy task as it further requires fine mapping using a larger segregating population with several markers. Additionally, large genome with repetitive and suppressed recombination regions such as centromeric and pericentromeric regions further complicates marker saturation and fine mapping (Appels et al., 2018; Choulet et al., 2014). Considering these challenges, there is a need for alternative and rapid gene cloning strategies, which have the potential to reduce time, labor, and resource requirements for rapid gene identification.

## 3 | ADVANCED GENE IDENTIFICATION TECHNIQUES: BEYOND MAP-BASED CLONING

### 3.1 | Harnessing NGS for efficient gene cloning

With recent improvements in genomics techniques, it is now possible to sequence extremely large genomes in a single day. For instance, the emergence of NGS technologies led to the development of exceptionally high-quality reference genome of hexaploid wheat (Appels et al., 2018; Zhu et al., 2021). Recently, researchers used NGS technology to explore the global genetic diversity of the hexaploid wheat by constructing wheat pan-genomes (Jiao et al., 2024; Walkowiak et al., 2020; White et al., 2024) (Table 2). NGS technologies were also employed in developing several high-density SNP genotyping arrays including, "Illumina Wheat 9K iSelect SNP array" (Cavanagh et al., 2013), "Wheat 15K SNP array" (Boeven et al., 2016), "Wheat Breeders 35K Axiom array" (Allen et al., 2017), "Wheat50K *Triticum* TraitBreed array" (Rasheed & Xia, 2019), "Wheat 55K SNP array," "Illumina Wheat 90K iSelect SNP genotyping array" (S. Wang, Wong, et al., 2014), "Axiom Wheat 660K SNP array," and "Axiom HD Wheat genotyping (820K) array" (Winfield et al., 2016). These SNP arrays facilitated genotyping of large number of wheat genotypes within a short period of time (Cavanagh et al., 2013; Gudi et al., 2023; S. Wang, Wong, et al., 2014; Winfield et al., 2016). Moreover, recent drastic decrease in the sequencing costs made SNP chips and sequencing-based genotyping platforms increasingly accessible for scientists and even wheat breeders. Overall, NGS has revolutionized the field of wheat genomics and accelerated wheat improvement.

Genome sequencing approaches were classified into two broad categories: (i) WGS and (ii) RR sequencing. WGS involves the sequencing of entire genome of an organism, including coding and noncoding regions. This helps in understanding the evolutionary history of a plant species as well as helps in identifying potential genes associated with targeted traits. However, routine utilization of WGS in wheat is still expensive. Therefore, an alternative, low-cost, and efficient genome sequencing techniques needs to be employed in routine genomic studies. One such approach involves the sequencing of enriched genomic regions referred as RR-sequencing. The RR-sequencing approaches can be utilized for better resolution without reference genomes (Van Tassell et al., 2008). Different types of RR-sequencing approaches

**TABLE 1** Overview of different gene cloning strategies used in wheat.

| Gene cloning technique | Principle | Strengths | Limitation or drawbacks | Examples |
|---|---|---|---|---|
| Map-based cloning (positional cloning) | Identification of causal gene through linkage mapping and fine mapping using large segregating populations | Classical method with proven success | Time consuming and require large mapping population with high-density markers | *Lr21* (Huang et al., 2003); *Lr34/Yr18* (Krattinger et al., 2009) |
| Targeted chromosome-based cloning via long-range assembly (TACCA) | Genome-complexity reduction via chromosome flow sorting with long-range linkage to assemble complex genomes | Reduced representation sequencing especially useful for large and complex genomes | Isolating similar sized chromosomes is difficult and need high-quality reference genome | *Lr22a* (Thind et al., 2017) |
| RenSeq | Targeted sequencing of resistance gene family (NBS-LRR or NLR) | Rapid and cost-effective enrichment of NLR sequences | Limited to NLRs only | *Sr22*, *Sr45* (Steuernagel et al., 2016) |
| MutChromSeq | Flow sorting and sequencing of mutated chromosome | Combines mutagenesis with chromosome sorting and sequencing | Similar sized chromosomes add complexity | *Sr43* (Yu et al., 2023) |
| MutRenSeq | EMS mutagenesis followed by RenSeq | Combines mutagenesis with NLR-enriched sequencing | Large mutant population needed; limited to NLRs only | *Sr22*, *Sr45* (Steuernagel et al., 2016) |
| MutRNASeq | RNA sequencing and mutant screening | Not restricted to NLR genes; can be used to identify mutations in all expressed genes | Difficult to differentiate different isoforms of a gene and capture low expressed genes | *Sr62* (Yu et al., 2022) |
| MutIsoSeq | Isoform sequencing (Iso-seq) of wild type parental lines and deep RNA sequencing of mutants | Captures full-length transcripts; enables accurate identification of splice variants | Difficulty in detecting low expressed genes | *Lr9/Lr58* (J. Wang, Li, Li, et al., 2023) |
| AgRenSeq | Modified GWAS using RenSeq and *k*-mer analysis | Exploits natural genetic diversity to clone multiple *R* genes simultaneously | Limited to NLR identification | *Sr46* and *SrTA1662* (Arora et al., 2019) |

Abbreviations: EMS, ethyl methanesulfonate; GWAS, genome-wide association studies; NBS-LRR, nucleotide-binding site leucine-rich repeat; NLR, nucleotide-binding leucine-rich repeats.

include: (i) general, that is, restriction-site associated DNA sequencing and transcriptome sequencing (RNA-seq) and (ii) targeted, that is, primer extension capture, on-array capture (NimbleGen array), and molecular inversion probes sequence enrichment (Good, 2012).

As discussed earlier, majority of wheat genome is repetitive in nature and sequencing such regions is not very useful (Van Tassell et al., 2008). On the other hand, capturing and sequencing the protein-coding region of the genome (i.e., exome) not only enables the efficient identification of targeted genes but also reduces the sequencing cost. Exome sequencing is a form of RR-sequencing, which simplifies the gene mapping and mutation calling processes by circumventing the challenges posed by repetitive regions of the genome unless the causal mutations are present in noncoding regions of the genome (Hussain et al., 2018). Various exome capture assays including, "Agilent SureSelect," "Illumina TruSeq Exome," and "NimbleGen SeqCap EZ Exome" have been proposed for wheat (Gardiner et al., 2019; He et al., 2019; Winfield et al., 2012). These assays utilize the capture probe sets derived from hexaploid wheat and its relatives. Applicability of the targeted genome sequencing approaches largely depend on the ability of capture probes to tolerate sequence mismatches. For instance, the NimbleGen SeqCap EZ system can tolerate up to 10% sequence mismatch, while the Arbor Biosciences Baits system can tolerate up to 20% mismatch (Burridge et al., 2022). Exome capture has been successfully employed in wheat for multiple purposes. For instance, the exome capture was employed to unveil the historic gene flow from wild relatives to cultivated accessions of hexaploid and tetraploid wheat (He et al., 2019; Mascher et al., 2013). Exome capture has proven to be a valuable tool for mapping and gene identification in wheat. For instance, exome capture was used to identify the natural variants of *Yr6* responsible for yellow rust resistance (Gardiner et al., 2016) and induced mutations (Hussain et al., 2018) in wheat.

GBS is another widely used cost-efficient RR-seq approach for genotyping several germplasm lines (Elshire et al., 2011). This approach has been successfully employed in identifying the potential genomic regions associated with agronomic

**TABLE 2** List of genotypes for which high-quality reference or scaffold level assembly available for common wheat (*Triticum aestivum* L.).

| Genotype | Origin | Growth habit | BUSKO score (%) | Assembly size (Gb) | Total/corrected gene number | Assembly type | References |
|---|---|---|---|---|---|---|---|
| Chinese spring | China | Spring | 97.6 | 14.6 | 106,914 | Reference quality assembly | (Appels et al, 2018; Zhu et al., 2021) |
| Fielder | USA | Spring | 97.9 | 14.7 | 151,846 | Reference quality assembly | (Sato et al., 2021) |
| Kariega | South Africa | Spring | 98.4 | 14.7 | 152,838 | Reference quality assembly | (Athiyannan et al., 2022) |
| Attraktion | Europe | Winter | 98.2 | 14.7 | 148,999 | Reference quality assembly | (Kale et al., 2022) |
| Arina*LrFor* | Switzerland | Winter | 98.2 | 14.6 | 120,967 | Reference quality assembly | (Walkowiak et al., 2020) |
| CDC Landmark | Canada | Spring | 98.1 | 14.4 | 119,027 | Reference quality assembly | (Walkowiak et al., 2020) |
| CDC Stanley | Canada | Spring | 98.3 | 14.5 | 119,377 | Reference quality assembly | (Walkowiak et al., 2020) |
| Jagger | USA | Winter | 98.3 | 14.5 | 119,461 | Reference quality assembly | (Walkowiak et al., 2020) |
| Julius | Germany | Winter | 98.3 | 14.4 | 119,135 | Reference quality assembly | (Walkowiak et al., 2020) |
| LongReach Lancer | Australia | Spring | 97.6 | 14.3 | 120,045 | Reference quality assembly | (Walkowiak et al., 2020) |
| Mace | Australia | Spring | 98.2 | 14.4 | 119,772 | Reference quality assembly | (Walkowiak et al., 2020) |
| Norin 61 | Japan | Facultative spring | 98.4 | 14.8 | 118,734 | Reference quality assembly | (Walkowiak et al., 2020) |
| PI190962 (spelt wheat) | Central Europe | Winter | 98.2 | 14.4 | 120,104 | Reference quality assembly | (Walkowiak et al., 2020) |
| SY Mattis | France | Winter | 97.8 | 14.9 | 120,827 | Reference quality assembly | (Walkowiak et al., 2020) |
| Cadenza | UK | Spring | – | – | – | Scafold level assembly | (Walkowiak et al., 2020) |
| Paragon | UK | Spring | – | – | – | Scafold level assembly | (Walkowiak et al., 2020) |
| Robigus | UK | Winter | – | – | – | Scafold level assembly | (Walkowiak et al., 2020) |
| Claire | UK | Winter | – | – | – | Scafold level assembly | (Walkowiak et al., 2020) |
| Weebill 1 | CIMMYT | Spring | – | – | – | Scafold level assembly | (Walkowiak et al., 2020) |
| BJ8 | China | Strong winter | 99.1 | 14.9 | 154,055 | Reference quality assembly | (Jiao et al., 2024) |
| MZM | China | Strong winter | 98.9 | 14.7 | 152,776 | Reference quality assembly | (Jiao et al., 2024) |
| XN6028 | China | Strong winter | 99 | 14.7 | 153,509 | Reference quality assembly | (Jiao et al., 2024) |
| Abo | China | Semi winter | 99 | 14.7 | 151,829 | Reference quality assembly | (Jiao et al., 2024) |
| NC4 | China | Spring | 98.9 | 14.6 | 151,701 | Reference quality assembly | (Jiao et al., 2024) |

(Continues)

TABLE 2 (Continued)

| Genotype | Origin | Growth habit | BUSKO score (%) | Assembly size (Gb) | Total/corrected gene number | Assembly type | References |
|---|---|---|---|---|---|---|---|
| YM158 | China | Semi winter | 99 | 14.7 | 153,162 | Reference quality assembly | (Jiao et al., 2024) |
| XY6 | China | Winter | 99 | 14.9 | 153,075 | Reference quality assembly | (Jiao et al., 2024) |
| AMN | China | Strong winter | 99 | 15 | 152,478 | Reference quality assembly | (Jiao et al., 2024) |
| JM47 | China | Strong winter | 99 | 15 | 154,828 | Reference quality assembly | (Jiao et al., 2024) |
| S4185 | China | Strong winter | 99 | 15 | 152,811 | Reference quality assembly | (Jiao et al., 2024) |
| CM42 | China | Semi winter | 98.9 | 14.7 | 151,806 | Reference quality assembly | (Jiao et al., 2024) |
| JM22 | China | Winter | 99 | 15 | 152,462 | Reference quality assembly | (Jiao et al., 2024) |
| KF11 | China | Winter | 99.1 | 14.9 | 151,964 | Reference quality assembly | (Jiao et al., 2024) |
| ZM366 | China | Winter | 99 | 14.8 | 153,198 | Reference quality assembly | (Jiao et al., 2024) |
| ZM16 | China | Winter | 99 | 15.1 | 154,524 | Reference quality assembly | (Jiao et al., 2024) |
| ZM22 | China | Winter | 99 | 15 | 153,111 | Reference quality assembly | (Jiao et al., 2024) |
| HD6172 | China | Strong winter | 99 | 15.1 | 154,470 | Reference quality assembly | (Jiao et al., 2024) |

traits, quality traits, disease resistance, insect resistance, and abiotic stress tolerance in wheat (Alipour et al., 2017; Gudi et al., 2024).

Though RR-sequencing approaches greatly reduce the sequencing cost, they may cause biasness in the genome representation as only the subset of genomic regions defined by restriction enzymes and their sites are sequenced. This results in the allele-specific dropout, uneven coverage across the genome, and over- or underrepresentation of some genomic regions. RR-sequencing also results in the distorted estimation of allele frequency. Moreover, biasness in genome representation is problematic in the introgression breeding and identifying alien introgression regions as introgressed chromosomal regions are usually differ in sequence composition and restriction site density, thereby underestimating the size and boundaries of introgression regions (Paun et al., 2019). These challenges can be overcome by employing more efficient RR-sequencing approaches, such as, targeted chromosome-based cloning via long-range assembly (TACCA). TACCA is a novel molecular technique that enables rapid cloning of genes from the complex genomes (Thind et al., 2017). TACCA utilizes flow cytometry to isolate the chromosome of interest, followed by long-range sequencing technologies to assemble the entire chromosome. TACCA guarantees that important genetic information will not lose during complexity reduction process, thereby overcoming a common limitation faced by other RR-sequencing approaches. This technique has been successfully utilized to clone leaf rust resistance gene, *Lr22a* located on wheat chromosome 2D (Thind et al., 2017). TACCA relies on the genetic recombination to delimit the candidate genomic region, which makes gene cloning very difficult if the gene of interest (GOI) lies in the reduced recombination regions like centromeric or pericentromeric region, structural rearrangements, and alien introgressions. In these cases, mapping interval or candidate genomic region may remain larger and often contain several, tightly linked genes. Therefore, researchers have developed a novel gene cloning approach that involves sorting and sequencing of the mutant chromosome and identification of causal variations by comparing it with the parental chromosome.

This approach is referred as MutChromSeq, which has been successfully employed to clone powdery mildew (*pm2*) (Sánchez-Martín et al., 2016), leaf rust (*Lr14a*) (Kolodziej et al., 2021), and leaf and stripe rust (*Yr87/Lr85*) (Sharma et al., 2024) resistance genes in wheat. MutChromSeq was also used with RNA sequencing to clone the leaf rust (*Lr9*) (Y. Wang, Abrouk, et al., 2023) and stem rust (*Sr43*) (Yu

et al., 2023) resistance genes in wheat. Despite these advantages, sorting individual chromosomes demands specialized instruments and technical skills, which is not accessible for everyone. Moreover, current chromosome sorting techniques are not efficient to isolate all 21 chromosomes of wheat, specifically when they are of similar size. (Vrána et al., 2000). Additionally, isolating high-molecular weight DNA from flow sorted chromosomes is challenging and the limited DNA quantity and quality results in the reduced sequencing depth and assembly. This limits the complete utilization of TACCA and MutChromSeq in wheat.

Therefore, alternative approaches such as resistance gene enrichment (i.e., RenSeq and its modifications including MutRenSeq, AgRenSeq, MutIsoSeq, and MutRNASeq) can be used to clone the disease resistance genes (Table 1). RenSeq is a targeted sequencing approach enable to discover the disease resistance genes by capturing and sequencing the nucleotide-binding site leucine-rich repeat. This strategy has been successfully employed in cloning clone stem rust (*Sr22*, *Sr26*, *Sr45*, and *Sr61*), stripe rust (*Yr5a*, *Yr5b*, and *Yr7*), and powdery mildew resistance (*Pm21* and *Pm1a*) genes in wheat (Steuernagel et al., 2016). AgRenSeq combines the RenSeq with *k*-mer-based GWASs to identify disease resistance genes. AgRenSeq was used to clone four (*Sr33*, *Sr45*, *Sr46*, and *SrTa1662*) stem rust resistance genes (Arora et al., 2019). MutIsoSeq integrates isoform sequencing of wild type parental lines and deep RNA sequencing of mutants to identify altered transcripts for candidate genes. MutIsoSeq was used to clone leaf rust resistance genes (*Lr9* and *Lr58*, both are same gene) (Hafeez et al., 2021; Y. Wang, Abrouk, et al., 2023). MutRNASeq combines RNA sequencing and mutant screening to identify disease resistance genes. MutRNASeq was used to clone the leaf rust resistance gene (*Lr47* and *Lr.ace-4A*/*Lr30*) in wheat (H. Li et al., 2023; J. Yang et al., 2025a).

Although RenSeq and its derivatives speedup the process of resistance gene cloning in wheat, they are still not a gold standard technique for cloning genes in wheat. This is owing to the dependence of RenSeq on the quality and diversity of bait libraries, which failed to capture diverse and novel nucleotide-binding leucine-rich repeats (NLRs), specifically those from wild relatives of wheat. Similarly, MutRenSeq need to develop and evaluate thousands of mutants, which is labor and time intensives, and miss genes not belonging to NLR family or those present in genomic regions with low mutation frequency. MutIsoSeq requires cost-intensive high-quality, long read RNA sequencing. Along with capturing targeted NLRs, MutIsoSeq may capture expressed isoforms or may miss genes with low expression. MutRNASeq also fails in differentiating different isoforms of gene and in capturing low expressed genes. Moreover, MutRNASeq faces difficulty in identifying causal variants from the low expressed genes or regions with complex and alternative splicing. Altogether, since RenSeq and its derivatives focus only on NLRs, it is not effective in cloning disease resistance genes encoded by other gene families. Moreover, these techniques are not suitable for cloning quantitative traits such as agronomic, quality, and abiotic stress tolerance, as they were designed to capture the major, single effect disease resistance genes. Quantitative traits are polygenic in nature controlled by multiple genes with small effect on phenotype, often outside the NLR boundaries, and do not produce clear phenotype in the mutant populations. Therefore, these techniques are not suitable for cloning genes controlling quantitative traits.

## 3.2 | GWAS: Cloning genes from natural populations

GWAS explores historical recombination and linkage disequilibrium (LD) to unravel the genetic architecture of complex traits using the natural population. It does not need biparental populations for identifying candidate genomic regions, thereby GWAS saves time and resources in developing biparental populations such as RILs. GWAS has been successfully employed in wheat to identify the potential genomic regions for yield and its component traits, disease and pest resistance, and abiotic stress tolerance (Gudi et al., 2024, 2025a, 2025b, 2025c, 2025d; Rajamanickam et al., 2024; Tanin et al., 2022; Tian et al., 2023; N. Zhang et al., 2023). It facilitated the cloning and characterization of genes associated with different agronomic and abiotic stress tolerance including *TaNAC071-A*, *TaPYL1-1B*, *DIW1/TaPP2C158*, *TaWD4b.1*, *TabHLH27*, *TaABF2*, *TaPYL9*, *TaCLE24b and TaDT1-A*$^{hapI}$, and *TaMAT1a-2B* (L. Du et al., 2024b; X. Liu et al., 2025; Y. Lu et al., 2025; Mao, Li, et al., 2022; Mao, Jian, et al., 2022; Tian et al., 2023; J. Wang, Li, Li, et al., 2023; D. Wang et al., 2024; W. Yang et al., 2025b; Y. Zhang et al., 2025). GWAS are generally based on SNPs and do not include all genetic variants. As a result, important genetic structural variants such as insertions, deletions, translocations, and copy number variations are not included in the standard SNP-based association analysis. Several studies suggested incorporating linkage mapping with GWAS to confirm identified QTL regions or genes as genomic regions solely identified by GWAS may have lower confidence. Moreover, multiple factors including larger LD blocks, breeding history, population size, phenotyping accuracy, genotyping resolution, population structure, kinship, and presence of rare alleles further reduce the efficiency of GWAS in pinpointing the candidate genes associated with trait of interest. To overcome these issues Voichek and Weigel (2020) suggested to identify genetic variants in the population by focusing on constant length of *k* from the original sequencing reads, called as *k*-mers (31 bp fragments) (Voichek & Weigel, 2020). *k*-mers from all the reads are then compared

among individuals to check for the presence/absence pattern. These *k*-mers can depict all-possible genetic variants including SNPs, insertions, deletions, and translocations in the population. The *k*-mer-based GWAS has been successfully employed to clone the agronomically important genes in wheat. For instance, Arora et al., 2019 combined the *k*-mer-based GWAS with R-gene enrichment sequencing referred as, "AgRenSeq," in *A. tauschii* association panel to clone four stem rust resistance genes (*Sr33*, *Sr45*, *Sr46*, and *SrTa1662*). Later, a modified *k*-mer-based approach has been developed and utilized to clone the stem rust (*SrTA1662*) and powdery mildew (WTK4) resistance genes in wild relative of wheat, *A. tauschii* (Gaurav et al., 2022). Despite of its importance, *k*-mer-based GWAS has its own limitations. Since, billions of *k*-mers need to be stored, counted, and used for association analysis, this demands for the huge computational resources requiring large memory and processing power. Till today, there are no efficient and user-friendly algorithms and programs are not developed to carry out the *k*-mer-based association analysis. Moreover, this technology demands for high sequencing depth in all germplasm lines, otherwise missing *k*-mers in low coverage genomic regions may identify spurious associations. Most of the time short *k*-mers may fail to map uniquely to large and repetitive genomes such as wheat, which further increases the chance of detecting spurious associations (Karikari et al., 2023). Therefore, there is a need for improving the *k*-mer-based GWAS by reducing cost of sequencing and identifying sequence variation underlying significant *k*-mers (Roberts et al., 2025). Moreover, pan-genome graphs can be utilized in identifying significant *k*-mers by allowing their alignment to the graph and the identification of haplotypes associated with phenotypic variation (Roberts et al., 2025). This will help in accelerating the identification of genes associated with key agronomic and quality traits along with abiotic stress tolerance.

### 3.3 | BSA and its improvements

BSA is the powerful genetic mapping technique used for identifying genomic regions associated with targeted traits (Altinkut & Gozukirmizi, 2003; Michelmore et al., 1991). In recent years, several advancements have been made in BSA to improve the resolution, speed, and precision of genetic mapping. For instance, BSA has been combined with SNP-chip genotyping (Aoun et al., 2017), NGS/WGS/GBS (i.e., BSA-seq) (Klymiuk et al., 2022), and RNA sequencing (i.e., BSR-Seq) (G. Lin, Chen, et al., 2022) to identify genomic regions associated with target traits. Most recently, several resistance genes in wheat such as a serine/threonine-protein kinase gene (i.e., *PmCH7087*) responsible for powdery mildew resistance and an NLR gene (i.e., *Lr42*) responsible for leaf rust resistance have been cloned using this approach (G. Lin, Chen, et al., 2022; Zhan et al., 2021).

Recently, BSA has been combined with NGS to identify causal mutations (i.e., MutMap, MutMap+, MutMapGap, and LumiMap) underlying altered phenotype. In MutMap, wild type plants are crossed with mutant plants to generate $F_2$ generation, and two bulk samples representing mutant and wild-type phenotypes were used for BSA. This technique has been successfully utilized to identify the genes responsible for various traits in wheat, including male fertility (*MS1*) (Z. Wang et al., 2017), stripe rust resistance (*YrJ22*) (C. Chen et al., 2022), and tiller number (*tin6*) (Schoen et al., 2023). MutMap+ is the improved version of MutMAP that eliminates the requirement of developing $F_2$ population. Instead, it relies on selfing $M_2$ plants to generate $M_3$ generation, which is then used for BSA. This technique has been used to identify ethyl methane sulfonate (EMS) mutants with reduced height and pale green leaves (*Hit9188*) in rice (*Oryza sativa* L.) (Fekih et al., 2013). MutMap and MutMap+ largely relies on a reference genome for sequence alignment. If causal mutations lie in the genomic regions that is not represented in the reference genome, then those mutations will not be identified. This problem can be overcome by *de novo* assembly of representative germplasm line to fill the gaps present in the reference genome. This approach is referred as MutMap-Gap and is used to clone the blast resistance gene (*Pii*) in rice (Takagi et al., 2013). Most recently, Kato et al. (2020) developed transgenic reporter plants containing the firefly luciferase (*LUC*) gene to identify mutant plants with altered luminescence patterns upon pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI). Once mutant plants were identified, MutMap technique was used to identify the causal SNP variants or mutants responsible for altered luminescence pattern (LumiMap) (Kato et al., 2020). As per our knowledge, none of the studies in wheat utilized MutMap+, MutMap-Gap, and LumiMap to identify the causal genes for any mutant. These advanced BSA techniques holds a great potential for studying altered phenotypes in wheat. Additionally, by leveraging these technologies, researchers can effectively identify and characterize mutant genes responsible for specific traits in wheat. However, despite their promise, MutMap and their improvements have several limitations, which needs to be considered before exploring them in wheat gene cloning programs. All these methods rely on developing and evaluating EMS-induced mutant populations for traits controlled by single, major effect genes. However, these techniques cannot be used for quantitative traits controlled by multiple genes with additive effect on phenotype, as it is very difficult to produce mutant lines with clear-cut phenotypes. Moreover, identifying mutant lines for the genes present in mutation recalcitrant regions is very difficult and may also require the development of even larger mutant populations, which is time, labor, and resource intensive. All these meth-

ods have demonstrated strong proof of concept success, but further standardization, improved analytical pipelines, and a reduction in sequencing costs will be required to ensure reproducibility across breeding environments and laboratories. Considering the applications and limitations, it is recommended to use these techniques cautiously for identifying causal genes in wheat.

## 4 | EXPLORING RECENT ADVANCES IN WHEAT FUNCTIONAL GENOMICS: TOOLS AND TECHNIQUES

Functional genomics is a branch of genomics, which helps in understanding functions of genes and their regulatory networks involved in plant growth, development, and responses to various biotic and abiotic stresses. Functional genomics tools have been broadly classified into (i) conventional approaches, (ii) genetic engineering approaches, (iii) genome editing approaches, and (iv) transient gene expression approaches.

### 4.1 | Conventional approaches

#### 4.1.1 | Mutagenesis: A powerful tool for gene characterization

As discussed in earlier sections, spontaneous and induced mutagenesis can be utilized for developing new traits or for the identification of genes responsible for targeted traits. Despite lower frequency of spontaneous mutations, several mutants with economic importance have been identified in wheat. For instance, the "green revolution" genes responsible for reduced plant height (i.e., *Rht-B1b* and *Rht-D1b*) are the spontaneous mutations with a single nucleotide substitution at N-terminal region of the protein involved in gibberellin signaling pathway (Pearce, 2021). Similarly, the dominant mutants of vernalization genes responsible for spring (*VRN1*) and winter type (*VRN2*) phenotypes in wheat and barley (*Hordeum vulgare* L) occurred spontaneously via a large deletion in the promotor sequence (Yan et al., 2003, 2004) or intronic regions (Fu et al., 2005). Additionally, the pistilloid condition in hexaploid wheat is the result of spontaneous mutation (Marais & Lombard, 1993). Artificial mutations are deliberately induced using either the physical or the chemical mutagenic agents. Physical agents such as x-rays, gamma-rays, UV rays, alpha and beta particles, and fast and thermal neutrons result in short or large deletions depending on type and intensity of the mutagenic agent. Physical mutagens, mainly X-rays were successfully utilized to identify different genes in wheat. A significant example of x-ray radiations to induce mutation is the homoeologous pairing gene, *ph1*, that enhanced the chromosomal pairing in the distant hybridization (Sears, 1977). Similarly, several genes were characterized by creating mutant libraries of 51,840 lines in *Arabidopsis* and 24,660 lines in rice using fast neutrons (X. Li et al., 2001; J. Wu et al., 2005).

Chemical mutagens including alkylating agents (i.e., EMS) base analogs (i.e., 5-bromouraci; 5-BU), intercalating agents (i.e., ethidium bromide), and deaminating agents (i.e., nitrous acid and hydroxylamine) usually generate point mutations. EMS is the most frequently used chemical mutagen to induce G/C to A/T transitions in plants. EMS was used to validate the functions of several genes including, waxy (Rawat et al., 2019), starch branching enzyme IIa (*SBEIIa*) (Botticella et al., 2011; Rawat et al., 2019), ADP-glucose pyrophosphorylase large subunit (*AGPL*) (Guo, Yan, et al., 2017), wheat GA 20-oxidase (*TaGA20ox1*) (King et al., 2015), leaf rust resistance genes (*Lr9*, *Lr42*, and *Lr58*) (G. Lin, Chen, et al., 2022; Y. Wang, Abrouk, et al., 2023), stem rust resistance genes (*Sr33*, *Sr43*, *Sr45*, *Sr46*, and *SrTA1662*) (Arora et al., 2019; Yu et al., 2023), stripe rust resistance genes (*Yr15* and *YrNAM*) (Klymiuk et al., 2018; Ni et al., 2023), and sucrose transporter (*SUT1*) (Rawat et al., 2019) (Figure 1). Most recently, Ni et al. (2023) have established sequencing trait-associated mutations (STAM) to clone a stripe rust resistance gene, *YrNAM* in wheat (Ni et al., 2023). STAM uses genome/transcriptome sequencing and de novo assembly of wild type genotype as a reference and employs transcriptome sequencing of relevant mutants for gene cloning. J. Wang, Li, Li, et al. (2023) used exome-capture sequencing of a mutant library consisting of 2090 mutant lines and identified the novel allelic variations in the genes responsible for several agronomic traits and stress tolerance (i.e., *Rht-D1*, *TaTB1*, *WFZP*, *SNAC1*, *DREB2B*, *CML16*, and *ZFP182*) (D. Wang, Li, Wang, et al., 2023). Despite the importance of mutation in functional validation of genes, its application is restricted to qualitative traits governed by single, major effect genes. However, creating and evaluating large mutant population for quantitative traits such as agronomic and abiotic stress tolerance is difficult as they are controlled by multiple genes with additive phenotypic effect. Moreover, mutation for these traits may not produce clear phenotype for these traits. Therefore, this is not a user-friendly method for functional validation of quantitative traits.

#### 4.1.2 | Allele mining unlocks the functions of genetic variants

Wheat has undergone several genetic modifications during crop evolution and domestication, which leads to the huge genetic diversity. For instance, wheat germplasm bank at CIMMYT, Mexico, conserves around ~170,000 accessions belonging to modern cultivars, land races, and crop wild relatives with rich source of unexplored genetic diversity

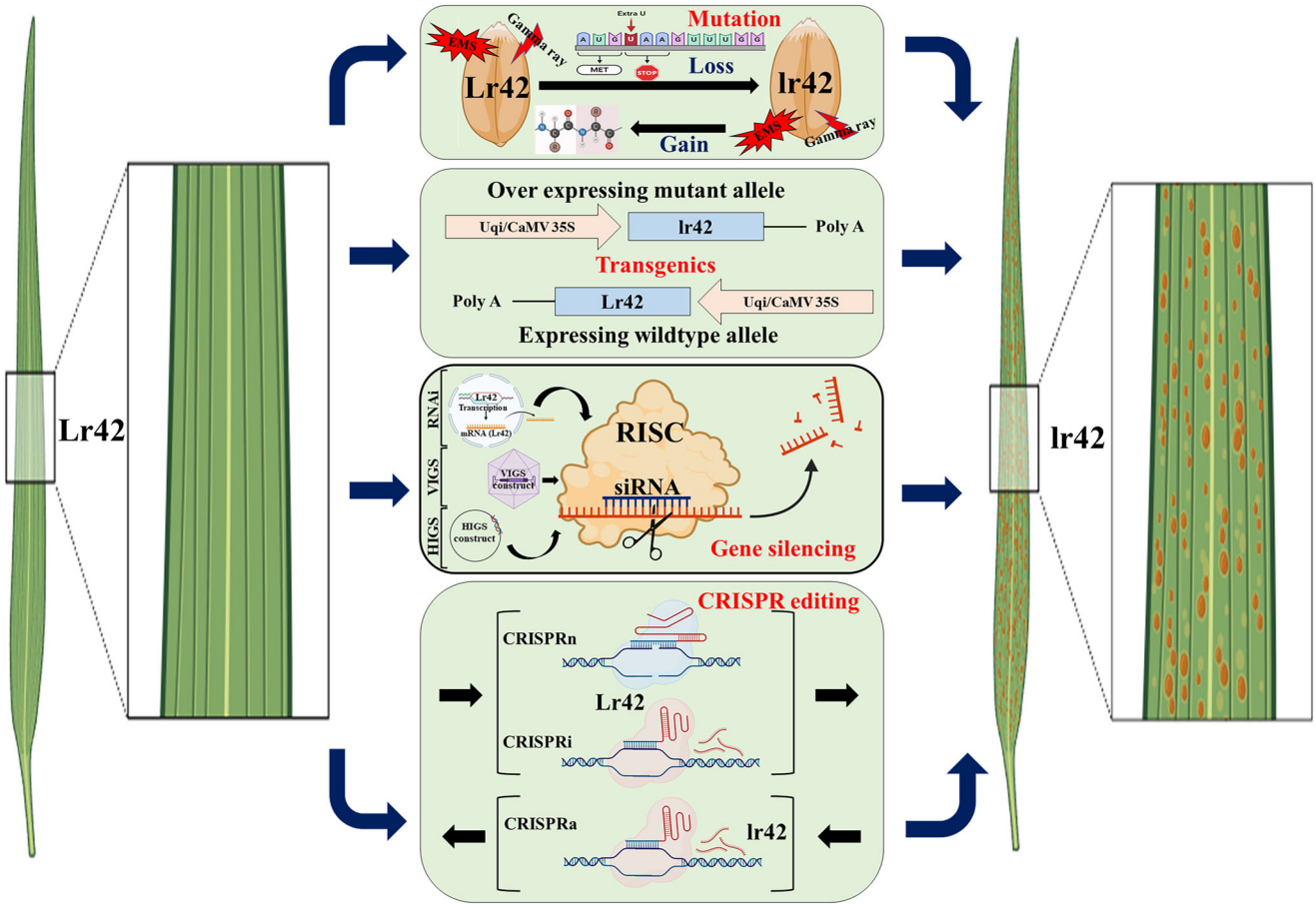


**FIGURE 1** Advanced functional genomics tools for validating the functions of genes: (a) inducing artificial mutagenesis using physical (gamma-radiation) or chemical mutagenic (ethyl methane sulfonate [EMS]) agents to produce loss of function or gain of function mutants; (b) transgenic approaches for overexpressing the targeted gene (either mutant or wild-type allele) using maize ubiquitin (Uqi) or 35S cauliflower mosaic virus (CaMV); (c) suppressing the gene expression either through RNA interreference (RNAi) or through transient-gene expression analysis (such as host-induced gene silencing [HIGS] and virus-induced gene silencing [VIGS]); and (d) utilizing efficient genome-editing tool (CRISPR-CAS) to validate functions of gene through CRISPR knockout (CRISPRn), CRISPR-based gene silencing (CRISPR-interference; CRISPRi), and CRISPR-based gene activation (CRISPR-activation; CRISPRa). Here, we took *Lr42*, a leaf rust resistant gene in wheat as a hypothetical example.

(Langridge & Waugh, 2019; Milner et al., 2019; Singh et al., 2022). Exploiting this genetic diversity is challenging as it requires identification and characterization of genomic regions responsible for target traits. "Allele mining" is a novel genomics approach for dissecting natural allelic variations present in crop germplasm pool and understand their functional relevance for the trait(s) of interest (Comadran et al., 2012; Singh et al., 2024). Additionally, allele mining facilitates the haplotype-based breeding to achieve higher genetic gain. Allele mining has been successfully used in wheat to identify and characterize several novel alleles associated with grain hardiness (*Pin-a* and *Pin-b*) (Giroux & Morris, 1997), powdery mildew resistance (*Pm3*) (Kaur et al., 2008; Yahiaoui et al., 2006), amylose biosynthesis (*Wx-A1* and *Wx-B1*) (Monari et al., 2005; Saito & Nakamura, 2005), vernalization response (*Ap1* and *PhyC*) (Beales et al., 2005), endosperm starch synthesis (*SSII*) (Shimbata et al., 2005), grain yellow pigment content (*PSY-1* and *PSY-2*) (W. Zhang & Dubcovsky, 2008), preharvest sprouting tolerance (*Viviparous-1*) (Xia et al., 2008), *Lr34/Yr18/Pm38* locus (Lagudah et al., 2009), abscisic acid receptor (B. Wu et al., 2022), and drought stress tolerance (*TaWD40-4B.1)* (Tian et al., 2023).

Allele mining can be achieved through targeting induced local lesions in genomes (TILLING), ecotype-TIILING (Eco-TILLING), and sequencing-based allele mining. TILLING and Eco-TILLING involves the identification of point mutations resulting from induced (as described in Section 4.1.1) and spontaneous mutations, respectively, for the targeted traits through heteroduplex analysis (Till et al., 2003). They can also be employed as reverse genetics approaches to validate the functions of genes (Krattinger et al., 2009; Periyannan et al., 2013). In the last decade, several TILLING populations have been developed in wheat to identify superior alleles associated with starch synthesis (*TaSSIVb-D*) (Guo, Liu, et al., 2017), powdery mildew resistance (*Mlo*) (Acevedo-Garcia

et al., 2017), high amylose content (Sestili et al., 2015; Slade et al., 2012), and kernel hardiness (W. Li et al., 2017). TILLING technique was also utilized to identify and combine the mutations at 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from A and D sub-genomes through two rounds of mutagenesis to develop glyphosate tolerant wheat (Moehs et al., 2021). Besides wheat, EMS-induced TILLING populations for A- (TILL-A) and D-genome (TILL-D) from the diploid progenitors, *Triticum monococcum* and *A. tauschii* were also developed (Rawat et al., 2012, 2018). These populations serve as important resources for gene discovery, gene validation, and allele mining.

Eco-TILLING was successfully employed to identify natural allelic variations for vernalization (*VRN-A1*) (L. Chen et al., 2011) and starch synthesis (*TaSSIV*) (Irshad et al., 2019) genes in wheat. Though TILLING and Eco-TILLING are valuable techniques of allele mining, they are resource-intensive, less efficient, and involve complex procedures requiring a considerable level of expertise. Moreover, the sensitive allele detection method of these techniques reduces the efficiency of allele mining. In contrast, sequence-based allele mining is a cost-effective and simple technique with more efficiency. This technique relied on PCR amplification and sequence comparison of the targeted gene from all germplasm lines to identify the novel allelic variants or haplotypes from the crop germplasm pool (Kumar et al., 2010). For instance, PCR amplification and sequence comparison led to the identification of three haplotypes for powdery mildew resistance at *Pm3* gene (Kaur et al., 2008), three haplotypes for leaf rust resistance at *Lr42* gene (G. Lin, Chen, et al., 2022), and two haplotypes for drought stress tolerance at *TaWD40-4B* gene (Tian et al., 2023). Despite of its key role in identifying the novel allelic variations, sequence-based allele mining fails to distinguish the homoeologous sequences in the polyploid species like wheat, thereby complicating the identification of novel alleles associated with targeted traits. Moreover, it fails to detect the rare variants or allelic variation for complex quantitative traits, specifically when the genetic diversity is less.

## 4.2 | Genetic engineering approaches in functional genomics: From transgenesis to gene-knockout

### 4.2.1 | Gain of function to study gene function

Traditionally, gene complementation or overexpression is being implemented in various model and crop plant species for functional validation. Native promotors of a gene and constitutive promoters are the most commonly used promotors to overexpress GOI in several crop species including wheat. Functional characterization using overexpression of GOI in wheat has been successfully used for FHB resistance (i.e., *α-1-purothionin*, *thaumatin-like protein 1*, and *β-1,3-glucanase*) (Mackintosh et al., 2007), stem rust resistance (i.e., *Sr33*, *Sr45*, *Sr46*, and *SrTA1662*) (Arora et al., 2019), leaf rust resistance (i.e., *Lr42*) (G. Lin, Chen, et al., 2022), stripe rust resistance (i.e., *YrNAM*) (Ni et al., 2023), powdery mildew resistance (i.e., *Pm24*) (P. Lu et al., 2020), drought stress tolerance (i.e., *TaSHN1*, *TaDTG6*, and *TaWD40-4B*) (Bi et al., 2018; Mei et al., 2022; Tian et al., 2023), heat stress tolerance (i.e., *TaHsfA6b*) (Poonia et al., 2020), and spike architecture and enhanced grain yield (i.e., *WAPO-A1* and *TaCol-B5*) (Wittern et al., 2022; X. Zhang et al., 2022) (Figure 1). This approach is useful in precisely studying the function of GOI without any confounding effect on other genes. However, in some cases overexpression may interfere with endogenous regulatory networks, causing nonspecific effects. In case of dominant mutation, mutant alleles can also be overexpressed using this approach. This results in the overaccumulation of nonfunctional proteins over wild-type proteins, and it may result in a mutant phenotype through dominant negative interaction. For instance, insertion of a mutant copy of *DES* gene (i.e., *mDES*) responsible for whole-seedling necrosis in the background of wild-type wheat plant resulted in co-segregation of *mDES* with seedling necrosis in the T1 generation (Jia et al., 2021).

Compared to other plant species, genetic engineering in wheat still lags behind largely because of multiple factors. First, wheat is a hexaploid species, where each gene is present in three copies, leading to a functional redundancy that complicates genetic transformation and gain of function studies. Second, most of the wheat germplasm lines are recalcitrant to in-vitro culture, plant regeneration, and genetic transformation, making the genetic transformation challenging (Shrawat & Armstrong, 2018). The efficiency of genetic transformation is generally low in wheat genotypes. However, some studies have reported high transformation efficiency of up to 70% and 90% in two wheat genotypes, Bobwhite and Fielder, respectively (Ishida et al., 2015; Pellegrineschi et al., 2002). Genotype dependency and lower transformation efficiency encouraged wheat researchers across the globe to carry research on improving the transformation efficiency. Efforts were also made for *in-planta* transformation approaches in wheat including (i) *Agrobacterium* mediated transformation after soaking seeds in water (Supartana et al., 2006), (ii) inoculating *Agrobacterium* to the incision made at the base of wheat seedlings (Zhao et al., 2006), (iii) inoculating *Agrobacterium* to the wounded apical meristems (Razzaq et al., 2011), (iv) *Agrobacterium* infection of immature embryos (i.e., in developing seeds) (Risacher et al., 2009), (v) floral dip method (Zale et al., 2009), and (vi) *Agrobacterium*-mediated microspore transformation (Rustgi et al., 2017). Even with these efforts, the transformation efficiency in wheat is quite low. In recent past, a set of different trans-

formation regulator genes, such as *GRF-GIF*, *Baby boom*, and *Wuschel*2 have been reported in different crops (Kong et al., 2020; Lowe et al., 2016). These regulators accelerate the growth of embryonic tissues in the culture media, thereby improving the plant regeneration and transformation efficiency. For instance, when *Baby boom* and *Wuschel2* genes were expressed in maize (*Zea mays* L.), the transformation efficiency was increased to 40% (Lowe et al., 2016). Similarly, plant regeneration and transformation efficiency were greatly influenced by expressing *Wuschel2* in sorghum (*Sorghum bicolor* L.) and *GRFs* or *GRF-GIF* in various dicot and monocot species (Che et al., 2022; Debernardi et al., 2020; Kong et al., 2020). Efforts were also made to identify and incorporate the *GRF-GIF* chimeric protein to improve the regeneration efficiency in wheat (Kong et al., 2020). Future studies should focus more on identification and integration of stable transformation regulator genes to enhance the transformation efficiency across different wheat genotypes.

#### 4.2.2 | Loss of function approach: RNAi

RNAi refers to the sequence-specific suppression of targeted genes induced by double-stranded RNA (dsRNA) molecules. RNAi mediated gene silencing can be achieved by (i) downregulating the expression of genes through promoter methylation (transcriptional gene silencing) and (ii) sequence-specific RNA degradation (post-transcriptional gene silencing [PTGS]). PTGS is triggered by dsRNAs molecules which are processed by Dicer-like proteins into siRNAs (small interfering RNAs with 21–24 bp length). Subsequently, these siRNAs bind to Argonaute proteins (AGOs) to form RNA-induced silencing complexes that target complementary RNAs for degradation or translational inhibition. RNAi has been used to silence the genes involved in vernalization (*VRN1* and *VRN2*) (Loukoianov et al., 2005; Yan et al., 2004), low amylopectin synthesis (*SBE-IIa*) (Regina et al., 2006), ethylene insensitivity (*EIN2*), photobleaching (*PDS*) (Travella et al., 2006), grain protein content (*GPC*) (Uauy et al., 2006), and gliadins (*γ-gliadins*) (Gil-Humanes et al., 2014) in wheat (Figure 1). The inefficient delivery of RNAi cassettes, presence of homoeologous copies in the genome, and the occurrence of off-targets further limit the use of RNAi in wheat functional genomics. The homoeologous copies of a gene across A, B, and D sub-genomes is considered a major factor during functional genomics studies in wheat. Masked and weak phenotypes are the results of the functional disruption of a single gene copy, due to compensation from its homoeologous copies, making it necessary to target multiple gene copies for the reliable functional validation (J. Li et al., 2021). Moreover, RNAi can lead to non-native expression patterns or pleiotropic effects that complicate interpretation. Therefore, careful selection of gene validation strategies is a must to account for gene family redundancy, genome complexity, and the need for robust and interpretable phenotypes in wheat.

### 4.3 | Genome editing: Opportunities for targeted gene modification

Recent breakthroughs in genome editing tools have opened new avenues for deciphering the functions of GOI, thereby facilitating the translation of genomic information into practical applications. Some of the genome editing tools includes (i) ZFNs; (ii) TALENs; and (iii) CRISPR-Cas9. ZFNs are the custom-designed DNA-binding proteins used in functional genomics studies to induce knockout mutations. ZFNs were used to edit acetohydroxyacid synthase gene to confer herbicide resistance in bread wheat (Ran et al., 2018). TALENs are the restriction enzymes which introduce double-strand breaks at the target site to produce loss of function or gain of functions mutations. For instance, a pair of TALENs were used to orchestrate precise mutations within the three homoeoalleles of wheat responsible for encoding *Mildew resistance locus* (*MLO*) proteins that resulted in powdery mildew resistance (Y. Wang, Cheng, et al., 2014). Similarly, TALENs were used to reactivate the nonfunctional copy of leaf rust resistance gene, *lr21*, but failed to restore resistance (Luo et al., 2019). This observed setback could potentially be attributed to the presence of editing footprints. Despite their potential applications, ZFNs and TALENs were less preferred genome editing tools owing to the intricacies involved in their design, limited target range, heightened off-target effects, and increased potential for cytotoxicity (specifically ZFNs). However, these problems were partially addressed by the more recent genome editing approach, CRISPR-Cas9.

CRISPR-Cas system can be utilized in three different ways in functional genomics studies including: (i) CRISPR knockout of targeted genes (CRISPRn), (ii) CRISPR-based silencing of targeted genes (CRISPR-interference [CRISPRi]), and (iii) CRISPR-based activation of targeted genes (CRISPR-activation [CRISPRa]).

CRISPRn utilizes Cas9 endonuclease to introduce double-stranded DNA breaks in the targeted genomic region, which undergoes error-prone non-homologous end joining repair to produce indels. Frameshift mutations resulting from these indels results in the knockout of targeted genes, making CRISPR-Cas9 ideal for loss of function studies. Knockout mutants were produced through *Agrobacterium* mediated transformation of CRISPR-Cas9 to validate the functions of *TaPDS* involved in carotene pathway (Howells et al., 2018), *TaDA1* and *TaDA2* involved in kernel development, *TaPinb* involved in grain hardiness, *TaNCED1* and *TaLPR2* involved in stress tolerance (S. Zhang et al., 2019), *ZnF* and *TaBKI1* genes involved in brassinosteroid signaling pathway (Song

et al., 2023), and *TaNHX2* and *TaGAD1* involved in drought stress (J. Li et al., 2024) in wheat. Additionally, gold particle coated, *in-planta* delivery (i.e., biolistic approach) of CRISPR-Cas9 were used to validate the functions of *TaMLO-A1* involved in powdery mildew resistance (Y. Wang, Cheng, et al., 2014); *α-gliadin* involved in low-gluten production (Sánchez-León et al., 2018); *TaGW2* involved in grain weight (Y. Zhang et al., 2018); *TaCol-B5* involved in spike architecture and grain yield (X. Zhang et al., 2022); *TaQsd1*involved in seed dormancy (Y. Liu et al., 2021); and *TaSD-A1*, *TaSD-B1*, and *TaSD-D1* involved in green revolution genes in wheat (Y. Kumagai et al., 2022) (Figure 1). Since, the transformation is a major challenge in wheat, scientists have recently developed a new technique to use wheat–maize hybridization to generate "transformation free" and "transgene free" genome editing in wheat. Using this approach, the CRISPR-Cas9 and gRNA targeting two wheat genes conferring susceptibility to FHB (*TaHRC*) and *Septoria nodorum* blotch/tan spot (*Tsn1*) were transformed in maize and used the transgenic pollen to pollinate wheat to produce the DH genome edited wheat (Karmacharya et al., 2023). Moreover, researchers developed the heritable transgene-free genome editing by grafting the normal wild-type scions with transgenic rootstocks (L. Yang et al., 2023). This technique still needs to be utilized in wheat.

Recent advances in genome editing have moved beyond the traditional CRISPR-CAS9-mediated double strand break to introduce single base pair change or specific nicks in the targeted genome. For instance, base editing uses catalytically dead Cas9 (dCas9) fused to deaminase to introduce single nucleotide change in the genome without creating double strand breaks (Komor et al., 2016). This helps in precise and accurate disruption of gene function. Similarly, prime editing is a next-generation, highly programmable gene editing tool, which uses reverse transcriptase with Cas9 nickase and prime editing guide RNA (pegRNA) creates targeted insertions or deletions (Anzalone et al., 2019). Efforts were made to standardize the base and prime editing protocols to produce knockout mutants in wheat (Gaillochet et al., 2023; Q. Lin, Zong, et al., 2020). In addition to these technologies, recent advances in large segment genome editing tools such as twin prime editing (Anzalone et al., 2022), RNA bridge recombinase (bridge RNA) (Durrant et al., 2024), and EyoCAST (Witte et al., 2025) allows the insertion, deletion, or rearrangement of extensive DNA sequences including complete gene sequence or large chromosomal regions with high accuracy. These genome editing tools can be introduced into wheat for enhancing wheat functional genomics. Besides gene knockouts, CRISPR-interference or activation (CRISPRi/a) can be utilized as alternative approaches to modulate the functions of targeted genes. For instance, stringent knockdown of coding and noncoding RNAs or the partial reduction in gene expression can be achieved by recruiting repressor domains to transcriptional start sites using dCas9 (Gilbert et al., 2013; Kampmann, 2018). Since CRISPRi acts on transcriptional start sites, it can be utilized in knocking down of specific transcripts or all isoforms of a gene. For instance, targeted expression of phytoene desaturase gene was regulated by constructing transcriptional activators and repressors by fusing activator and repressor domains to the C-terminus of dCas9, respectively, in wheat (Zhou et al., 2022). Studies were also employed to regulate the gene expression through DNA acetylation or methylation using CRISPR-Cas9 (Hilton et al., 2015; Laufer & Singh, 2015).

Despite these advances, genome editing for functional gene validation in wheat still face several limitations. As discussed earlier, hexaploid nature of wheat with three copies of a gene create functional redundancy and masks the phenotypic effect of single gene edits. Genotype-dependent transformation and poor regeneration efficiencies remain a bottleneck in wheat. Achieving transgene-free edits is at initial stage in wheat and moreover, it is labor and time intensive. Since CRISPRi/a need gRNAs (sgRNAs) to target the transcription start sites or promotor regions. The lack of PAM sequences or chromatin modifications at transcription start sites or promotor regions make them inaccessible to CRISPR-mediated regulation. Additionally, targeting genes with multiple start sites in promotor regions may not be achieved through sgRNA (Ford et al., 2019; Kampmann, 2020). Generation of off target edits and incomplete repression or activation of function further complicates the functional analysis. Therefore, before going to use CRISPR-mediated functional validation, it is crucial to consider the genomic complexity of wheat. Moreover, it is necessary to make further improvements in CRISPR-based functional genomics tools for understanding the functional and cellular aspects of targeted genes in wheat.

## 4.4 | Transient gene expression analysis: Fast functional genomics tool

Virus-induced gene silencing (VIGS) is reverse genetics approach used to study the function of GOI by transiently downregulating or silencing its expression in plants, especially in crop species, where establishing stable transformants is challenging. In this approach, DNA fragments from plant genome are integrated into viral genomes (Cakir et al., 2010; M. Kumagai et al., 1995; Ratcliff et al., 1997). Barley stripe mosaic virus (BSMV) is most used for VIGS in wheat. VIGS based on BSMV has been successfully employed in functional genomics studies to validate the functions of wheat genes involved in leaf rust resistance (*Lr21*, *Lr42*, and *Yr87/Lr85*) (G. Lin, Chen, et al., 2022) (Sharma et al., 2024; Scofield et al., 2005), high-molecular-weight glutenin subunit (*1Bx14*) (Ma et al., 2012), *Zymoseptoria tritici* resistance and susceptibility (Lee et al., 2015), FHB resistance (*TaArf2-like*) (W. Chen et al., 2016), drought tolerance (*CPK34*) (G.-Z.

Li et al., 2020b), Rubisco activase isoforms (Perdomo et al., 2024), phytoene desaturase (*TaPDS*) (Garg et al., 2024), etc. Researchers have also used brome mosaic virus–based VIGS vector to validate the functions of phytoene desaturase (*TaPDS*) and phosphate2 (*TaPHO2*) genes in wheat (Y. Wang et al., 2021). Similar to VIGS, plant pathogen genes can also be silenced by *in-planta* gene silencing also known as host-induced gene silencing (HIGS). HIGS involves expressing dsRNA molecules complementary to pathogen genes in plants to precisely silence the targeted pathogen genes. HIGS has been successfully employed in characterizing pathogen genes causing powdery mildew (*Avra10*) (Nowara et al., 2010) and rusts (*MAPK*, *cyclophilin*, *calcineurin*, *PsCPK1*, and *Pgt-IaaM*) (Panwar et al., 2013; Qi et al., 2018; Yin & Hulbert, 2018). While the transient systems, like apoplast assays and VIGS allow rapid screening, their effects may vary across tissues but do not always reflect the stable genetic outcomes. Additionally, because these systems do not create stable, heritable genetic changes, the observed effects may not accurately reflect agronomic performance in field conditions. In some cases, VIGS can also trigger responses unrelated to the target gene, further complicating interpretation. Therefore, results obtained from transient assays often require confirmation through stable transformation, or multiplex editing approaches to reliably validate gene-trait relationships.

## 5 | CONCLUSION AND FUTURE OUTLOOK

Significant advances in wheat genomes have occurred since the beginning of 21st century. The development of improved genomics and functional genomics technologies, high-resolution mapping approaches together with lower sequencing costs, has made gene cloning in wheat easier than it was 20 years ago. These advances helped in identifying key genes responsible for improved disease resistance and agronomic performance. Furthermore, improvement in genetic engineering tools such as advanced transformation and plant regeneration efficiency, allowed the functional validation of multiple wheat genes. Additionally, advanced genome editing tools including base editing and prime editing helped in making precise changes in the targeted genomic regions. Furthermore, strategies such as pollinating transgenic pollen from maize with wheat enabled the development of transgene free wheat, even in wheat accessions reluctant to gene transformation and plant regeneration. Despite these efforts, the practical challenges such as complexity of polyploid genome, functional redundancy among homoeologous gene copies, and difficulties in achieving efficient transformation still limit the applications of these approaches in large scale breeding programs. Moving forward, integrating multi-omics and pan-omics (such as pan-genomics and pan-transcriptomics) data with multiplex genome editing, high-throughput phenotyping, and accelerated generation advancement systems such as speed breeding offers a promising path for efficient gene discovery and trait improvement. Establishing standardized pipelines, shared genomic resources, and collaborative networks are essential for translating functional genomics into practical wheat improvement, enabling gene discovery, validation, and deployment into wheat breeding programs.

## AUTHOR CONTRIBUTIONS

**Santosh Gudi**: Data curation; funding acquisition; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing. **Khizar Razzaq**: Conceptualization; formal analysis; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing. **Jatinder Singh**: Formal analysis; investigation; methodology; visualization; writing—review and editing. **Luis Del Rio Mendoza**: Funding acquisition; project administration; resources; supervision; writing—review and editing. **Mohamed Khan**: Funding acquisition; project administration; resources; supervision; writing—review and editing. **Rajeev Gupta**: Funding acquisition; project administration; resources; supervision; writing—review and editing. **Upinder Gill**: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—original draft; writing—review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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