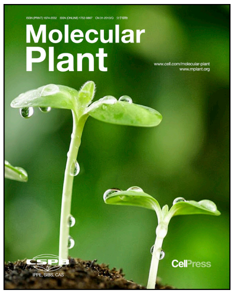

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Barley2035: A decade vision on barley research and breeding

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ABSTRACT

Barley (*Hordeum vulgare* ssp. *vulgare*) is one of the oldest founder crops in early human civilization, and has been widely dispersed around the globe to supply human life through livestock feeding and brewing industries. It has been used in innovative research of cytogenetics, biochemistry, and genetics since the early half of the 20th century, facilitated by its mode of reproduction through self-pollination, its true diploid status which has contributed to the accumulation of a plethora of germplasm and mutant resources. Coming to the era of molecular genomics and biology, a multitude of barley genes and their involved regulatory mechanisms have been uncovered and functionally validated, providing the paradigm for equivalent studies in other Triticeae crops. This review features the advancements over the past decade in barley research, mainly regarding genomics and genomics-assisted germplasm exploration, genetic dissection of developmental and adaptation associated traits, as well as the complex dynamics of yield and quality formation. For the coming decade, the perspective of integration of these innovations in barley research and breeding is promising. Barley is proposed as a reference in Triticeae crops for new gene discovery, functional validation and molecular mechanism dissection. The application of precise genome editing as well as genomic prediction and selection, further enhanced by artificial intelligence-enforced tools and applications, is expected to boost barley improvement, in order to efficiently meet the evolving global demands for this important crop.

Keywords: Barley, genomics, pre-breeding, gene editing, hybrid breeding, triticeae

SHORT SUMMARY

This review summarizes a decade of advancements in the genetics, genomics and biology of barley, one of the founder crops in early human civilization. The authors present their perspectives on the future research directions and enhancement of this important crop through utilizing extensive germplasm resources coupled with cutting-edge methodologies to satisfy the evolving demands of sustainable agriculture for the forthcoming decade.

INTRODUCTION

Barley (*Hordeum vulgare* ssp. *vulgare*) ranks as the fourth most important cereal crop globally, both in terms of harvested area and production (**FAO dataset**; https://www.fao.org/faostat/en/#data). Its early maturation, robustness and adaptability to various environments underpin barley's success as a globally grown crop (**Figure 1A**) (reviewed by (Campoli and von Korff, 2014; Fernandez-Calleja et al., 2021; Liu and Jones, 2024; Nevo and Chen, 2010; Song et al., 2015; Xu and Chong, 2018)). It stands out as the singular crop being cultivated at the remarkable altitudes reaching 4,700 meters on the Qinghai-Tibetan plateau. Breeding efforts have constantly increased its yield potential (**Figure 1B**). As a vital component of the global feed and malting industries, barley also serves as a dietary staple in certain regions (**Figure 1C**). It is a significant player in international trade (**Figure 1D**-**E**). It became a model species for crop research in the 20th century (**Figure 1F**), due to its self-pollinating nature, its true diploid genome, a haploid set of seven large chromosomes, and the rich availability of germplasm resources and genetic stocks (Lundqvist, 2014).

Barley was domesticated approximately 12,000 years ago from its wild ancestor (*Hordeum vulgare* ssp. *spontaneum*) in the Fertile Crescent (Harris, 2001). It's likely one of the earliest cultivated plants, serving as a cornerstone for the development of early human societies. There are morphological variants at the taxonomical traits, such as the row type of the spike (two-rowed *vs.* six-rowed) and the adherence of the hulls (lemma and palea) to the mature caryopsis (hulled *vs.* naked grain) (Komatsuda et al., 2007; Taketa et al., 2008). Barley was also among the first plants to benefit from techniques such as mutagenesis (Stadler, 1928), double-haploid (DH) production (Kasha and Kao, 1970), hybrid breeding (Ramage, 1965), genetic mapping by help of molecular markers (Graner et al., 1991), and genetic transformation to produce transgenic plants (Wan and Lemaux, 1994). The earliest commercial cultivars obtained through mutation breeding, 'Pallas' and 'Mari', were approved in 1958 and 1960, respectively (reviewed by (Lundqvist, 2014)), and the first barley hybrid cultivar, 'Hembar', was marketed in the late 1960s (reviewed by (Ramage, 1983)). Since the 1990s, barley research and breeding have been further advanced by adopting innovations from genetics and genomics. Map-based cloning and genome-wide association studies (GWAS), which rely on the principles of genetic linkage and linkage disequilibrium, respectively, have become prevalent in barley gene discovery (Buschges et al., 1997; Cockram et al., 2010; Rostoks et al., 2006). Numerous functional genes/loci have been identified (reviewed by (Hansson et al., 2018); **Supplemental Table 1**; **Figure 2**).

The large and complex genome of barley (approximately 4.5 Gb with over 80% repetitive DNA elements, based on genome sequencing) was a challenge to whole genome sequencing and assembly, especially when compared to the relatively simple genomes of model species like Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*). High-quality genomes, pangenome and pan-transcriptome resources are available (IBSC, 2012; Jayakodi et al., 2020; Mascher et al., 2017; Mascher et al., 2021; Jayakodi et al., 2024), along with newly-established databases (**Supplemental Table 2**), holding a promise for innovation in future barley research and breeding. The progress made in barley genomics has set a precedent for similar studies in other temperate cereal crops that possess large and complex allopolyploid genomes, such as common wheat (*Triticum aestivum*) (Appels et al., 2018), durum wheat (*Triticum turgidum*) (Maccaferri et al., 2019), and oats (*Avena sativa*) (Kamal et al., 2022; Peng et al., 2022). Genebank genomics (McCouch et al., 2013), the systematic sequence-based genotyping of entire genebank collections, early featured in wheat (Sansaloni et al., 2020) and barley (Milner et al., 2019), offers a solution to bridge the gap between germplasm resources and future research and breeding in crops (Mascher et al., 2019).

Here, we would like to showcase the achievements of the past decade in barley research, and on this backdrop provide a perspective of opportunities and challenges for barley research and application of the upcoming decade. Benefiting from genomics-assisted rapid gene characterization and precise genome editing, barley may gain importance more than ever before as a diploid model within Triticeae to explore and characterize genes at large scale, especially the recessively inherited, whose function is easily obscured in a polyploid organism.

ADVANCEMENTS IN THE PAST DECADE

Genomes and databases

As a pure diploid species, barley has consistently been at the forefront of innovation in genomics, with each advancement marking a significant step forward (IBSC, 2012; Jayakodi et al., 2020; Mascher et al., 2017). Building upon the early foundational work of cytological maps ((Kunzel et al., 2000); reviewed by (Houben et al., 2018)), genetic maps (Graner et al., 1991; Varshney et al., 2007), and bacterial artificial chromosome (BAC) libraries (Schulte et al., 2011), the International Barley Genome Sequencing Consortium (IBSC), due to the high costs associated with traditional Sanger sequencing technologies, initially focused on sequencing gene-enriched regions (Schulte et al., 2009). The rapid development of the Illumina Hi-seq platform and the adventure of next-generation sequencing (NGS) technology (Shendure and Ji, 2008), transformed genomic studies for species with large genomes like barley. By combining BAC-end sequencing with the Sanger method, BAC shotgun sequencing with the Roche 454 approach, and whole-genome shotgun sequencing with the Illumina platform, along with transcriptome sequencing and genetic anchoring, the IBSC unveiled a physical map (IBSC, 2012). This map spanned 4.98 billion nucleotides, with 76% genetically anchored and included the annotation of 26,159 high-confidence genes, marking it as the barley draft genome.

Various techniques have been developed to enhance the anchoring of NGS-contigs (International Wheat Genome Sequencing, 2014; Mascher et al., 2013a) and to improve the scaffolding of these contigs (Lieberman-Aiden et al., 2009). In 2017, a chromosome-scale assembly of the first barley reference genome was achieved, covering approximately 95% (4.79 Gb) of the estimated barley genome size, with 95% (4.54 Gb) of the assembled sequences assigned to specific chromosomes (Mascher et al., 2017). Moreover, additional barley draft genomes or high-quality assemblies representing diverse accessions have been assembled as well (Dai et al., 2018; Jiang et al., 2022a;

Liu et al., 2019; Pan et al., 2023; Sakkour et al., 2022; Sato et al., 2021; Xu et al., 2021; Zeng et al., 2015). Based on the germplasm diversification revealed by Genotyping-by-sequencing (GBS) of over 22,000 genebank accessions (Milner et al., 2019), 20 genetically distinct representatives, including landraces, cultivars, and one accession of wild barley, were selected to construct the inaugural version of the barley pangenome (Jayakodi et al., 2020). This effort uncovered a plethora of large inversion polymorphisms, some of which were identified as imprint of irradiation-induced mutation at the early time.

The latest release of the barley genome reference is 'Morex.v3' that combined long-read sequencing and improved algorithms in sequence assembling (Mascher et al., 2021). Besides archiving in the general hubs such as 'NCBI', 'EBI' and 'Graingenes', there are many specialized web-based tools developed for exploring barley genomic resources (**Supplemental Table 2**). The 'IPK Galaxy Blast Suite' facilitates sequence BLAST against the latest barley reference genome 'Morex.v3', and the genome assemblies of 76 wild and domesticated barleys which constituted the second version of barley pangenome (Jayakodi et al., 2024), as well as *H. bulbosum* pangenome (Feng et al., 2024), a relative that is considered as the secondary gene-pool of cultivated barley (Wendler et al., 2014). 'BARLEX' is an invaluable tool for searching annotated genes within a specific version of the barley reference genome. This includes the gene annotation, coding sequence, gene IDs in versions of the 'Morex' genome assembly, and full-length cDNA sequencing of the Japanese cultivar 'Haruna Nijo' (Matsumoto et al., 2011). It also lists molecular markers from barley SNP arrays (Bayer et al., 2017) that can target the gene and provides a link to the gene's expression profile. The newly developed 'PanBARLEX' tool enables exploration of genes and orthogroups across the pangenome. 'BRIDGE' is a web portal to barley genebank genomics, offering passport information and phenotypic data, with SNP browser and VCF export functions. 'BaRTv2.18' is currently the most comprehensive and highly resolved reference transcript dataset for barley, derived from the European two-rowed spring barley cultivar 'Barke' (Coulter et al., 2022). A pan-transcriptome for barley has been assembled, capturing diverse tissue-specificity from the 20 genotypes used for the version 1 of the pangenome (Guo et al., 2024). The transcriptome datasets 'BaRTv2.18' (Coulter et al., 2022) and the multifaceted RNA-seq database 'BarleyExpDB' (Li et al., 2023) represent the current active resources for barley gene expression data.

Germplasm and genomics-enhanced germplasm exploration

The global distribution and millenia-long adaptation to diverse climates, latitudes and altitudes of barley has fostered a rich intraspecific diversity in growth habits and plant morphology. Most of this diversity has been preserved in genebanks worldwide, each with more or less detailed passport information for traceability (reviewed by (Mascher et al., 2018)). For access to genebank-preserved barley germplasm and genetic stocks, a summary of major genebank websites is provided in previous literature (Mascher et al., 2018). Barley was reported with over 485,000 accessions in genebanks worldwide, including cultivated barley, wild barley, and other wild *Hordeum* species, along with numerous genetic stocks, and breeding lines (Knüpffer, 2009). Approximately 280,000 are documented in the 'Genesys PGR' (https://www.genesys-pgr.org/) with passport information available. To represent the maximum genetic diversity with a manageable number of accessions, the concept of a core collection has emerged, facilitating phenotypic evaluation in controlled environments. For example, the international barley core collection (BCC) comprises about 1,500 accessions from diverse regions and institutions (Knüpffer and Hintum, 2003). Research communities have also established other core collections for categories, such as wild barley, landraces, cultivars or breeding lines (**Supplemental Table 3**). The development of nested association mapping (NAM) populations or multi-parent advanced generation inter-cross (MAGIC) populations has increased polymorphism while simplifying the genetic background compared to natural association mapping populations. For example, the NAM population 'HEB-25' that incorporates genetic diversity from 25 wild barley accessions, as well as MAGIC populations that each derived from multiple founder genotypes, have been instrumental in mapping both qualitative and quantitative traits (Dang et al., 2022; Hautsalo et al., 2021; Maurer et al., 2015; Nice et al., 2016; Sharma et al., 2018; Vatter et al., 2018). *H. bulbosum* represents the secondary gene pool for barley due to its incomplete crossing barrier. Since the 1990s, efforts have focused on developing introgression lines with *H. bulbosum* chromosomal segments (reviewed by (Pickering and Johnston, 2005)). The advance of NGS technology has enabled the development of high-density molecular markers for precise delineation of introgression segments and targeted selection (Wendler et al., 2017; Wendler et al., 2015; Wendler et al., 2014).

Moreover, a unique resource in barley is a series of introgression lines with 881 mutants at morphological or physiological traits, which are backcrossed into a common genetic background, the two-rowed cultivar 'Bowman' (Druka et al., 2011; Hansson et al., 2024). These genetic stocks with passport information are preserved in the Barley Genetic Stock Collection (Aberdeen, Idaho, USA; https://npgsweb.ars-grin.gov/gringlobal/search) and at NordGen (Alnarp, Sweden; https://bgs.nordgen.org/index.php), and are identified with accession ID prefixes ‘GSHO’ or ‘NGB’, respectively. Additionally, a number of independent mutagenesis populations have been developed over the past two decades (**Supplemental Table 4**). These mutants expanded the genetic diversity in addition to natural variants of barley (Dockter and Hansson, 2015), and have accelerated identification of functional genes using molecular methods (Jiang et al., 2022a; Mascher et al., 2014; Szurman-Zubrzycka et al., 2023).

The information on sequence variations has spurred the development of high-throughput genotyping arrays (Bayer et al., 2017; Close et al., 2009), enabling efficient evaluation of genetic diversity among natural populations and germplasm collections (Munoz-Amatriain et al., 2014; Pasam et al., 2014). The agronomically-important traits can be linked to specific sequence variations (Sharma et al., 2018; Wiegmann et al., 2019). Additionally, complexity-reduced re-sequencing methods like genotyping-by-sequencing (Poland et al., 2012) and exome-sequencing (Mascher et al., 2013b) have become cost-effective for large populations, significantly facilitating the rapid isolation of novel genes to decipher environmental adaptation (Russell et al., 2016), and enhancing genomic-assisted genebank management (Milner et al., 2019).

Morphology and development

The absence of a crossing barrier between cultivated barley and its wild progenitor ssp. *spontaneum* allows for the development of genetically segregating populations between the crop and the non-domesticate, making barley an ideal model for studying the change of crop architecture during domestication. Wild barley possesses a brittle rachis causing the spikelets or grains to thresh freely as they approach maturity, whereas cultivated barley has a non-brittle rachis, ensuring bulk harvests. Classical forward genetics approaches have identified two tightly linked genes controlling rachis firmness, named *Brittle and tough rachis 1* (*Btr1*) and *Btr2* (Pourkheirandish et al., 2015). The recessive non-brittle variants (either *btr1* or *btr2*) were independently selected in geographically separate regions. Both genes encode uncharacterized proteins, and remain to be revealed of their molecular mechanisms.

It is interesting that there exists a special type of germplasm, the Tibetan weedy barley (*Hordeum vulgare* ssp. *vulgare* f. *agriocrithon*), which was first reported by Aberg (Aberg, 1938). It has the brittle rachis trait characteristic of wild barley, but produces six-rowed spikes. Although temporarily discussed in the literature (Dai et al., 2012; Pourkheirandish et al., 2018; Tanno and Takeda, 2004), the hypothesis of Tibet representing one of the centers of barley domestication could be rejected with evidences from latest genomic analysis (Gao et al., 2024; Guo et al., 2022). It was demonstrated that the brittle rachis trait in Tibetan weedy barley occurred as a recombination event between an Eastern and a Western cultivated barley haplotype reconstituting the wild-type allelic state at the two closely linked brittle rachis loci *Btr1* and *Btr2*, respectively, resulting in 6-rowed brittle barley.

Given the close relationship between spike architecture and grain yield potential, understanding of spike morphogenesis has made significant strides in recent years (reviewed by (Gauley and Boden, 2019; Koppolu and Schnurbusch, 2019)). Genetic dissection has shown that the row-type is due to multiple independent variations at discrete loci, including six-rowed spike 1 (*vrs1*) to *vrs4* (Bull et al., 2017; Komatsuda et al., 2007; Koppolu et al., 2013; van Esse et al., 2017; Youssef et al., 2017), *INTERMEDIUM-C* (*int-c* or *vrs5*) (Ramsay et al., 2011), and *int-m* (Zhong et al., 2021). The spike branching mutant *com1.a* had been isolated, encoding a grass-specific TCP transcription factor (Poursarebani et al., 2020). Moreover, the spikelet is the fundamental unit of the barley inflorescence, and several independent genes, when deficient, causing a multi-ovary mutation, have been cloned (e.g., *mov1*, *mov2*, *mov3*, and *mov5*, which convert stamens into pistils to varying degrees) (Selva et al., 2021; Selva et al., 2023; Sun et al., 2024; Yang and Tucker, 2021).

The barley genetic stocks are rich with variants in spike density, such as the *dense spike* (*dsp*), *erectoides* (*ert*), *zeocriton* (*Zeo*), and *laxatum* (*lax*). The allelic mutants *Ert-r* and *Zeo1/Zeo2/Zeo3* were attributed to variations in the transcription factor gene *APETALA2* (*AP2*), which is a major determinant in the spike density variation within natural populations (Houston et al., 2013). The gene *lax-a*, responsible for the relaxed spike phenotype, encodes a homolog of the Arabidopsis *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* transcription factors (Jost et al., 2016), while its paralogous gene *HvCul4* regulates the tillering of barley plants (Tavakol et al., 2015).

The ‘Green Revolution’ has brought significant changes in plant architecture, with semi-dwarf varieties being widely adopted, improving culm architecture from tall and slender to short and sturdy necessary to avoid culm breakage and plant lodging of high-yielding modern varieties grown under high fertilizer input (Hedden, 2003; Zhang and Zhang, 2003). In modern varieties of malting barley, various semi-dwarf genes/alleles *sdw1.d*, *sdw1.c/denso*, *ari-e.GP*, *Zeo2* and *ert-k.32* have been successfully implemented in breeding programs (Dockter and Hansson, 2015; Zakhrabekova et al., 2023). Semi-dwarf gene, *Semidwarf 1* (*sdw1*), regulates culm length via the GA pathway, encoding gibberellin 20-oxidase 2 (GA20ox2), with different recessive allelic variants used in various breeding programs (Kuczynska et al., 2013; Teplyakova et al., 2017; Xie et al., 2024; Xu et al., 2017). For example, the allele *sdw1.d* originated from X-ray mutagenesis and was initially released in 1965 as cv. 'Diamant'. This variant has been used for breeding of over 160 registered cultivars as new malting barley in several countries (Dockter and Hansson, 2015). The *uzu1.a* allele, carrying a substitution in the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Chono et al., 2003), is prevalent in traditional cultivars in East Asia and present food barley varieties in Japan, but absent in modern barley varieties for feed and malting. The barley ortholog of rice *DEP1* encodes a subunit of the heterotrimeric G protein (Huang et al., 2009). The barley semi-dwarf mutant *breviaristatum-e.GP* (*ari-e.GP*) exhibits a dense and erect spike, a short, sturdy culm and short, globe-shaped grain. Complementary transformation with a functional *DEP1* allele can restore culm length in *ari-e* plants (Wendt et al., 2016). The barley cultivar 'Golden Promise', carrying the loss-of-function allele *ari-e.GP* at *DEP1,* was a staple of the British malting industry and has the respective modified plant architecture. Numerous other short-culm mutants are preserved in the Nordic Genetic Resource Center (NordGen, Alnarp, Sweden), representing a valuable resource for expanding the genetic toolkit of the Green Revolution (Dockter and Hansson, 2015; Hansson et al., 2024; Lundqvist, 2014), and offering potentials of improving fertilizer use efficiency as has been demonstrated in rice and wheat (Sun et al., 2014; Li et al., 2018; Song et al., 2023).

Regulators modulating anther tapetum development (Hua et al., 2023), pollen maturation (Amanda et al., 2022), synapsis and crossover during meiosis (Colas et al., 2016), and male gamete production (Qi et al., 2019) have been identified crucial for barley fertility. Genes that control the trichome development and awn roughness have also been isolated (Milner et al., 2019; Jayakodi et al., 2024). A variety of chlorophyll mutants are primarily governed by Mendelian genetics and have long served as phenotypic markers. The molecular mechanisms behind some of these regulatory pathways have been elucidated, greatly enhancing our understanding of chlorophyll biosynthesis and chloroplast biogenesis, the essential processes in green plants (Li et al., 2019;

Overlander-Chen et al., 2024; Stuart et al., 2021; Taketa et al., 2021).

Biotic and abiotic stress

The powdery mildew resistance gene *mlo*, cloned from barley nearly three decades ago (Buschges et al., 1997), has become a cornerstone in the field of plant disease resistance (Kusch and Panstruga, 2017). Its significance spans beyond the Triticeae family to the entire plant kingdom, with the *mlo*-mediated resistance mechanism that has been recognized as a universal defense strategy against powdery mildew in various plant species (Kusch and Panstruga, 2017). Utilizing the genomics approach 'MutChromSeq' (Sanchez-Martin et al., 2016), the first barley leaf rust (*P. hordei*) resistance gene *Rph1* was cloned, highlighting the crucial role of coiled-coil nucleotide-binding site leucine-rich repeat (NLR) receptor proteins in resistance to fungal pathogens (Dracatos et al., 2019). Several other leaf rust resistance genes, including *Rph3*, *Rph7*, and *Rph15*, have been successfully cloned (Chen et al., 2021; Chen et al., 2023a; Dinh et al., 2022).

Significantly, the molecular characterization of *Rphq2* from *H. bulbosum* and *Rph22* from cultivated barley demonstrated that both host and non-host resistance to leaf rust can be achieved by modifying orthologous genes (Wang et al., 2019b). Both genes encode lectin receptor-like kinases and they are orthologous. *Rphq2* conferred stronger resistance to *P. hordei-bulbosi* (adapted) than *P. hordei* (non-adapted), while for *Rph22* the reverse was observed. Similarly, a tandem genetic module consisting of an exocyst subunit Exo70 and a receptor kinase Pur1 was identified, conferring non-host resistance against wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*) in barley (Holden et al., 2022). Research into the host specificity of soil-borne pathogens among *Hordeum* wild species would expand the gene pool for the exploitation of non-host resistance (Jin et al., 2023).

In comparison to the numerous fungal resistance genes that have been identified (Dracatos et al., 2023), the cloning of resistance genes to viruses in barley has been relatively limited. The most widespread viral pathogens infecting barley are aphid-transmitted *Barley yellow dwarf virus* (BYDV) and *Cereal yellow dwarf virus* (CYDV), as well as soil-borne plasmodiophorid-transmitted Bymoviruses *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) (Ordon and Kuhne, 2014). The complexity of greenhouse inoculation, phenotypic scoring, and the extended pathogenesis period of viral diseases, have increased the time required for map-based cloning. To date, only two resistance genes against BaYMV/BaMMV have been successfully cloned:

rym4/*rym5*, which encodes the eukaryotic translation initiation factor 4E (eIF4E) and is involved in the translation initiation of viral precursor proteins (Kanyuka et al., 2005; Stein et al., 2005), and *rym1*/*11*, which encodes the protein disulfide isomerase-like 5-1 (PDIL5-1) and is hypothesized to function as a chaperone in the folding of viral proteins (Yang et al., 2014a; Yang et al., 2014b). The knockout of either *eIF4E* or *PDIL5-1* homologs in common wheat conferred resistance to *Wheat yellow mosaic virus* (WYMV) (Kan et al., 2022; Kan et al., 2023), a member of the *Bymovirus* genus, indicating a conserved mechanisms of viral infection in barley and wheat. There are at least 14 other resistance loci against BaYMV/BaMMV that have been genetically mapped but are yet to be cloned (Jiang et al., 2020). Several genes conferring resistance/tolerance to BYDV or CYDV, have been reported but neither have been isolated yet (Ordon and Kuhne, 2014; Pidon et al., 2024).

Herbicide tolerance is a critical issue in the cultivation of barley, with the development of the imidazolinone (IMI)-tolerant barley varieties being a key objective for the barley breeding companies from Australia (https://grdc.com.au/; through searching for 'breeding for imidazolinone tolerant barley varieties: industry issues and concerns'). These efforts have mainly been focused on induced mutations in the acetohydroxyacid synthase (ALS) gene (Lee et al., 2011). Given the emergence of herbicide-resistant weeds like *Hordeum murinum* ssp. *glaucum* (Ngow et al., 2020; Shergill et al., 2016), there is an urgent need to explore new target components in barley beyond ALS.

Barley and its wild progenitor are well adapted to a variety of abiotic stresses through resistance and avoidance mechanisms. Seed dormancy, as an example, serves as a protective mechanism against adverse conditions, while a weak dormancy can lead to pre-harvest sprouting caused by late-season rainfall. Two genetic loci controlling the strength of seed dormancy have been identified: QTL for seed dormancy 1 (*Qsd1*) and *Qsd2*. *Qsd1* is associated with a single amino acid substitution in an alanine aminotransferase (AlaAT) and evolved from early domesticated barley in the southern Levant region (Sato et al., 2016). *Qsd2* encodes the mitogen-activated protein Kinase Kinase 3 (MKK3) (Nakamura et al., 2016), a conserved orthologous protein of the wheat pre-harvest sprouting (*PHS1*) gene (Torada et al., 2016). An amino acid substitution in MKK3 that increases the dormancy was selected in the cultivars of East Asia where there is a high risk of pre-harvest sprouting due to overlapping rainy and harvest seasons.

Due to its tolerance to a range of abiotic stresses, barley has been proposed as a model for understanding plant adaptability to climate change (Dawson et al., 2015). Significant progress has been made, including the early successful cloning of a boron transporter gene using map-based cloning (Sutton et al., 2007). Recently, numerous studies have highlighted barley's remarkable resilience to soil acidity (Feng et al., 2020; Ma et al., 2016), metal or trace element toxicity (Hayes et al., 2015) (Leplat et al., 2016; Wu et al., 2015), nutrient deficiency (George et al., 2014) (Avila-Ospina et al., 2015; Quan et al., 2019), drought (Fan et al., 2015; Honsdorf et al., 2014; Muzammil et al., 2018; Xiong et al., 2025), waterlogging (Mendiondo et al., 2016; Wang et al., 2024), and temperature fluctuations (Francia et al., 2016; Ingvordsen et al., 2015; Kruszka et al., 2014; Li et al., 2021a; Tondelli et al., 2014).

The response and tolerance to salinity in barley has received high attention (Munns and Tester, 2008). Through allele mining and transgenic studies, the high-affinity potassium transporters HKT1;1 (Qiu et al., 2011; Han et al., 2018), and HKT1;5 (Hazzouri et al., 2018; Huang et al., 2020) have been identified to exert significant influence on the salinity tolerance. The transcription factor CBF4 (Wu et al., 2011) and the vacuolar H+-pyrophosphatase AVP1 (Schilling et al., 2014) have been implicated in salinity tolerance mechanisms. The integration of multi-omics approaches has enabled the mapping of a comprehensive salinity stress response atlas for barley root and shoot (Shen et al., 2016; Shen et al., 2018). However, the isolation of specific genes responsible for salt tolerance through forward genetics seems to remain challenging, with many QTLs (Fan et al., 2016; Huang et al., 2008; Liu et al., 2017; Saade et al., 2016), such as *Nax3* (Shavrukov et al., 2010) and *Nax4* (Rivandi et al., 2011), still awaiting their molecular characterization.

Yield and quality

Although grain yield, yield stability and quality are the ultimate targets in crop improvement, the complexity of their quantitative inheritance has made genetic and molecular dissection more challenging. In barley, grain yield is determined by the number of spikes per unit area, the number of grains per spike, and grain weight, with the former two being closely related to plant architecture. Barley gene *CCT MOTIF FAMILY 4* (*HvCMF4*) has been identified as positive regulator for spike development, and mutations in this gene lead to an increase in primordia death and pollination failure, eventually resulting in a reduced number of grains per spike (Huang et al., 2023). Grain weight is closely associated with grain size and the process of grain filling. Grain size is determined by grain length, width, and thickness. A recent review of the genetic mapping and orthologous mapping of genes related to barley grain size over the past 20 years identified approximately 200 QTLs and 270 marker-trait associations (MTAs) through linkage and whole-genome association analyses, respectively (Hong et al., 2023). These loci are distributed across all seven chromosomes, with considerable redundancy detected in different studies.

Map-based cloning of QTLs associated with barley grain weight has not yet been reported; however, some genes identified for other traits have been found to simultaneously affect grain size or grain weight. For instance, the naked caryopsis gene *nud* significantly reduces grain weight compared to hulled grains (Knudsen et al., 2022; Milner et al., 2019; Taketa et al., 2008), and the six-rowed spike (*vrs*) genes lead to a smaller grain in the lateral spikelet (Bull et al., 2017; Komatsuda et al., 2007; Koppolu et al., 2013; van Esse et al., 2017; Youssef et al., 2017). Genes that regulate spike density, such as *Erectoides-r*/*Zeocriton1* (Houston et al., 2013; Shoesmith et al., 2021) and *qSRN1* (Fan et al., 2023), also impact grain weight. A genome-wide association study of grain yield associated traits in European barley cultivars has detected an overlapping interval comprising a putative ortholog of rice *GW2* and a QTL hotspot on barley chromosome 6H (Xu et al., 2018). Knockout of *GW2* in the barley variety 'Paustian' verified its functional conservation in affecting grain width and thousand grain weight (Knudsen et al., 2022). Mutations in plant architecture regulators like *HvDEP1* would reduce grain length and weight but increase grain width, the latter grain phenotype being preferred by the malting industry (Wendt et al., 2016). Disrupting the cellulose synthase-like gene *CslF6* could eliminate the (1,3;1,4)-β-glucan in the grain, which is desirable for the distilling and brewing industries; however, this also inadvertently leads to a decrease on thousand grain weight and total grain yield. Other mutants with amino acid substitutions in this gene showed no significant yield penalties (Knudsen et al., 2022).

Grain quality in barley is defined by its end use. The animal feed prefers a high protein-to-starch ratio, while the malting industry requires specialized starch as well as protein content, and human consumption favors nutrients like β-glucan (Fan et al., 2017). Previous genetic mapping studies have identified several associations, but the cloning of causal genes and their implementation in breeding programs are rather limited. However, reverse-genetics approaches like TILLING (Targeting Induced Local Lesions IN Genomes) and genome editing have yielded variants with significant modifications to the content/composition of grain storage components (Sparla et al., 2014; Yang et al., 2024). A recent study has shown that disrupting one out of several enzyme genes in the hydroxynitrile glucosides (HNGs) biosynthetic pathway could fully eliminate HNGs as a source of ethyl carbamate formation in whisky production (Jorgensen et al., 2024).

Barley grains exhibit a rich diversity in pigmentation. The accumulation of anthocyanins, such as delphinidin 3-glucoside or cyanidin 3-glucoside, results in blue or purple aleurone, respectively (Jia et al., 2020; Strygina et al., 2017; Xu et al., 2023), while the presence of black lemma and pericarp (*Blp*) is due to melanin accumulation (Li et al., 2024a). These pigments play a crucial role in protecting grains from oxidation during storage and enhancing nutritional value. Barley germplasms from the Qinghai-Tibet Plateau in China, which often have blue or purple grains, are thought to offer protection against ultraviolet radiation (Xu et al., 2023). Genetic diversity analysis suggests a monophyletic origin for black barley, possibly evolved from a merger of European and Ethiopian lineages (Long et al., 2018). Recently, the blue aleurone loci *Blx1* (Jia et al., 2020) and *Blx2* (Xu et al., 2023) have been fine-mapped with candidate genes proposed, and the *Blp1* locus has been associated with complex genomic structural variations among barley germplasms (Li et al., 2024a). Further dissection and genetic validation of these candidate genes are warranted.

Given the growing global demand for high-quality forage, barley is cultivated in many regions for its whole plant biomass (Bai et al., 2024). The defensive alkaloid gramine in barley leaves serves as a natural insect repellent but detracts from its palatability for ruminants. By leveraging comparative genomics and gene-editing technology, the gramine biosynthesis pathway in barley has been recently elucidated and genetically modified (Dias et al., 2024).

FUTURE PERSPECTIVE

Harnessing diversity for future barley improvement

Modern agricultural practices, focused on high-yielding and input-dependent monoculture cash crops, have been linked to greenhouse gas emissions, loss of biodiversity and soil and land degradation (McCouch et al., 2013). These issues have become a significant concern for governing bodies such as the European Union and have promoted the development of strategies for the transformation of the agricultural sector towards environmental sustainability (Boix-Fayos and de Vente, 2023). Moreover, 'The European Green Deal' passed by European Commission intends to reducing carbon emissions by at least 55% by 2030. Enhancing crop diversity is recognized as crucial for food security (Siddique et al., 2021; Zsögön et al., 2022), and it is also essential for breaking the domestication bottleneck faced by those founder crops (Meyer and Purugganan, 2013).

As shown in a century-scale experiment with barley, natural selection drives emergent genetic homogeneity (Landis et al., 2024). Breeder's selection has boosted the genetic uniformity, raising an urgent need to diversify the genetic pool of elite barley varieties. For example, the European winter barley protects against BaYMV/BaMMV mainly relying on *rym4* or *rym5*, and it has resulted in a risk as the virus strains evolve rapidly (reviewed by (Jiang et al., 2020)). Through a survey of globally collected barley landraces, an allelic variant of *rym4*/*rym5* was detected with broad-spectrum resistance against multiple viral strains (Shi et al., 2019). Exploiting and stacking additional resistance loci is another strategy to protect barley growing in different regions (reviewed by (Jiang et al., 2020)), especially deploying those resistant genes from the virus non-adaptive wild relatives (Jin et al., 2023; Pidon et al., 2021).

The malting barley breeding history in Australia offers an encouraging example. As an isolated continent, Australian's barley cultivation history is relatively brief and the genetic diversity among historical cultivars is comparatively lower than in other areas, such as Asia. The extensive use of exotic germplasm resources collected globally has significantly boosted the genetic diversity in modern Australian varieties, which is approximately 12.5% higher on genetic diversity than that of the historical cultivars (Hill et al., 2021). Selecting and enriching the pre-existing genetic variants from the European and African gene pools has enabled barley to adapt to the Australian environment (Hu et al., 2023b). Given that frequent occurrence of extreme heat and drought has severely impacted barley production and the global beer supply (Xie et al., 2018), breeding new barley varieties with climate resilience is now a top priority for the future.

The international barley research community is diligently working to decode the genetic diversity present in barley germplasm resources through cutting-edge genomic technologies. Implementing sequencing-based methodologies, such as genotyping-by-sequencing (GBS) (Milner et al., 2019) or sequencing of multiplied PCR-amplified fragments like BarPlex v1.1 (Gao et al., 2024), generated sequence-based bio-digital information. Seventy-six wild and domesticated genomes have been sequenced to date using single molecule long-read sequencing methods, while 1,315 genomes have been analyzed with short-read sequences (Jayakodi et al., 2024). These datasets enable us to understand the genetic basis of enormously important barley traits and how humans have adapted them to meet local demands and specific environmental conditions. With the robust pipeline for genome assembly and variation analysis, coupled with the declining cost of sequencing, it is anticipated that an array of barley germplasm panels, breeding lines and wild relatives would have their genomes sequenced in the near future, eventually providing informed data to assist breeders for selecting germplasm with optimal diversity in the breeding programs.

Rapid gene isolation to accelerate future pre-breeding

Unraveling the functional genes behind target traits, in comparison to their linkage markers, would better support an efficient utilization of those elite germplasms in pre-breeding. The identification of functional genes can be accomplished through two principal methodologies: forward genetics, which traces the genetic basis from observed phenotypes (**Figure 3A**), and reverse genetics, which investigates the phenotypic outcomes of known genetic elements (**Figure 3B**). Over three decades of genetic research have yielded a significant number of genetic loci and QTLs associated with various traits (Reviewed by (Hansson et al., 2024); Summarized by Barley Genetics Newsletter, https://wheat.pw.usda.gov/ggpages/bgn/; **Supplemental Table 1**). While in comparison to the number of the annotated coding genes in the latest barley reference genome (Mascher et al., 2021), the identified loci represent just the tip of the iceberg. The map-based cloning strategy can provide genes and corresponding molecular markers for precise selection in breeding programs. Although a gene locus delimited to a small genetic interval usually corresponds to a larger physical distance harboring a number of annotated genes, it's encouraging that, with the advancements in genomics technology, the time and labor cost have decreased dramatically (Mascher et al., 2014).

For those genes located at chromosomal regions with suppressed recombination (e.g. proximal to the centromere), the integration of genomics with mutagenesis can expedite gene isolation in many cases. These mutants produced by physical or chemical treatments, are highly homogeneous to their parental lines. A segregating population derived from crossing a mutant with its corresponding parental line can substantially reduce the number of unlinked polymorphisms, simplifying the identification of trait-related genes. The use of high-throughput whole-genome sequencing (WGS) for simultaneous mutant and wild-type bulk sequencing allows for rapid candidate gene identification irrespective of its position on chromosomes (Sun et al., 2024), thus eventually streamlining the positional gene isolation process (**Figure 3A**). Alternatively, reverse genetics for rapid gene isolation is also becoming viable. Mutations at specific genes or loci identified through TILLING can be quickly validated using corresponding mutants for particular trait(s) (**Figure 3B**). Established methods for exploring induced mutations include conventional

TILLING (**Supplemental Table 4**) and amplicon-seq (Jiang et al., 2022a), FIND-IT (Fast Identification of Nucleotide variants by droplet DigITal PCR; Knudsen et al., 2022), exome-captured complexity-reduced sequencing (Krasileva et al., 2017; Wang et al., 2023a; Xiong et al., 2023), and whole-genome sequencing (Jung and Till, 2021). Barley has an extensive collection of mutants, with for instance over 12,000 mutants/variants preserved at the Nordic Genetic Resource Center (Hansson et al., 2024; Lundqvist, 2014). Many of them have been confirmed to be allelic (Lundqvist, 2014), and systematically sequencing of these well characterized mutant collections would accelerate functional analysis of genes in barley.

Once a gene-trait association is validated, mining for elite allelic variations in germplasm panels allows for the development of functional markers to precisely select elite alleles (**Figure 3C**). Marker-assisted selection, whenever combining with classical backcrossing strategies and speed breeding techniques that optimize the temperature and photoperiod to shorten the life cycle (Watson et al., 2018), or by increasing population size with the aid of genome-wide high-density markers to select desirable traits while simultaneously minimizing genomic segments from the donor line (**Figure 3D**), will enhance the timing and accuracy of future pre-breeding processes.

In contrast to developmental and morphological traits, for which a number of regulatory genes have been identified (**Supplemental Table 1**), many agronomically important traits that are inherited quantitatively, such as yield components, stress responses, and nutrient use efficiency, remain less well understood. Phenotypic assessment might be a primary constraint in the identification of QTLs with minor effects. We hypothesize that with state-of-art facilities for precision phenotyping, the combination of natural germplasms with genetic resources having a uniform genetic background (e.g. induced mutants, complemented transformation plants, or gene edited plants) (e.g. *HvCMF4* that positively regulates the number of grains per spike; Huang et al., 2023), will enable the cloning of accessibility of minor QTLs, which are crucial for trait enhancement.

Genetics to Biology: Exploring molecular basis and gene networks

Over the past three decades, a number of barley functional genes have been cloned through forward-genetics strategies (**Supplemental Table 1**). Unraveling the molecular basis and functional regulatory networks of these genes is crucial for accelerating their optimal application in breeding practice. Transcriptomic analysis is a widely implemented strategy to depict the regulatory network.

Recent studies through this strategy have shed light on the comprehensive regulatory landscapes of barley floret (Chen et al., 2023b), inflorescence (Huang et al., 2023; Shanmugaraj et al., 2023; Shen et al., 2023; Thiel et al., 2021), and grain development (Kovacik et al., 2024). The pan-transcriptome datasets, which encompasses multiple tissues from 20 genotypes, have revealed the diversification of co-expression module-tissues correlations, offering a comprehensive gene-expression atlas (Guo et al., 2024). These reference transcriptomes may refine data analysis and interpretation for similar studies in the future.

Cis-regulatory elements are pivotal in the transcriptional gene regulation, and a recent study has demonstrated the potential of modifying cis-elements, such as promoter sequences, to shape quantitative traits in crop plants (Wang et al., 2021). Epigenetic regulation, often through DNA/histone methylation and demethylation, is a form of cis-regulation at the chromation structural level and has been proven to be crucial and widely present in barley development and morphogenesis (Zhang and Zhu, 2024). Various NGS-based methods for capturing cis-elements have been developed (e.g., Chromatin immunoprecipitation next-generation sequencing (ChIP-seq), DNA affinity purification sequencing (DAP-seq), Assay for targeting accessible-chromatin with high-throughout sequencing (ATAC-seq)), which are expected to systematically map and profile regulatory elements such as promoters, enhancers, and silencers, as well as associate them with agronomically important traits (Kovacik et al., 2024; Schmitz et al., 2022). An epigenomic analysis has provided an overview of chromatin states in the barley genome using chromatin ChIP-seq technology (Baker et al., 2015). The development of lateral spikelet is likely controlled by epigenomic regulation, as the row-type determinant gene *vrs3* encodes a histone demethylase that regulates chromatin state and the transcriptional activity of other *vrs* genes (Bull et al., 2017). The miRNA172-mediated quantitative variation in the abundance of the transcription factor AP2 serves as another example of post-transcriptional regulation via cis-element variations (Nair et al., 2010; Houston et al., 2013; Patil et al., 2019; Shoesmith et al., 2021). Nucleotide susbstituions of *AP2* mRNA result in modifications of its cleaveage efficiency by miR172, leading to variations on accumulations of AP2 protein in barley inflorescences, consequently, diverse phenotypes.

Trans-regulation through protein-protein interaction or protein-nucleotide interaction can be further investigated using a range of molecular techniques such as yeast one hybrid (Y1H) or two-hybrid (Y2H), co-immunoprecipitation (Co-IP), pull-down, electrophoretic mobility shift assay

(EMSA), and microphysics-derived homogeneous time-resolved fluorescence (HTRF) as well as surface plasmon resonance (SPR). For instance, the AP2 protein physically interacts with HvMADS1 to regulate the awn/lemma development via synergistically activating downstream targets (Zhang et al., 2024a). The rapid development of protein 3D modeling enhanced by AI and its-based prediction of molecular interactions (Tsuchiya et al., 2022) may reduce the effort required to validate candidate interactions.

In addition, employing multi-omics approaches might accelerate the dissection of these complex traits; for instance, metabolomics and microbiomics have demonstrated their utility in studying the interactions between plants and their abiotic or biotic environments in crop species, including barley (Zeng et al., 2020; Zhang et al., 2019).

Genome editing and genomic selection assisting future barley improvement

With knowledge of functional genes and their associated regulatory networks, genome editing technologies, like CRISPR/Cas9, are revolutionizing the next-generation of plant breeding by enabling precise and predictable modifications into crops to achieve desired traits (Gao, 2021). In plants, following the first report of editing the wheat powdery mildew susceptibility gene *MLO* (Wang et al., 2014), which is orthologous to the barley powdery mildew resistance gene *mlo* (Buschges et al., 1997), genome editing has made significant strides over the past decade (Li et al., 2024b; Zhu et al., 2020). In barley, CRISPR/Cas9-mediated genome editing has been widely applied to modify traits such as disease resistance (Cheng et al., 2022; Hoffie et al., 2021; Hoffie et al., 2022; Kis et al., 2019), plant architecture and chloroplast development (Cheng et al., 2023; Li et al., 2019; Xie et al., 2024), growth habit (Antonova et al., 2024; Hisano et al., 2022), grain properties (Garcia-Gimenez et al., 2020; Jiang et al., 2022b; Sparla et al., 2014; Yang et al., 2022; Yang et al., 2024; Yang et al., 2020), and nitrogen use efficiency (Karunarathne et al., 2022). These studies demonstrate the potency of this method and its significant potential for targeted improvement of elite barley varieties.

The genotype specificity in *Agrobacterium*-mediated transformation currently remains a limiting factor, with the majority of researches being conducted on a limited number of genotypes, such as the spring cultivar ‘Golden Promise’, and winter cultivar ‘Igri’. Co-transformation with specific developmental regulators (Wang et al., 2022) or using different types of explants (Yong Han et al., 2020) has shown some improvement in transformation and regeneration efficiency. Engineered

Agrobacterium strains suppressing host defense responses have increased transformation efficiency in several crop species (Raman et al., 2022). Alternatives to *Agrobacterium*, such as nanoparticle (Lv et al., 2020; Zhao et al., 2017) or viral RNA-based delivery systems (Li et al., 2021c), may overcome the genotypic constraints of the transformation system (Chen et al., 2022).

It's worth to note that the current policy for managing genome-editing or transformation crops remains strictly held in most nations worldwide (Gao, 2021). Global scientists continue to push for progress, proposing a science-based regulatory framework for genome-edited crops (Huang et al., 2016). Since genome editing is not a single technology but a molecular toolbox, a comprehensive, one-fits-all regulatory approach may be unlikely to achieve. Instead, a tiered regulatory system should be used to accommodate both existing and future technologies (Gao, 2021).

Besides manipulating a few particular gene loci, genomic selection is facilitated by constructing a predictive model from a training dataset, which is then used to examine a large number of individuals in a testing set based on the estimated breeding values (Crossa et al., 2017; Fu et al., 2022). It offers an opportunity to efficiently select superior genotypes, thus hastening the breeding cycle. In barley, the early achievements in genomics have enabled the development of high-throughput genotyping arrays with considerable representation of the genome (Bayer et al., 2017). Genomic prediction has also demonstrated its effectiveness in supporting the targeted selection of accessions with high breeding value by testing hybrid performance across germplasms (Sommer et al., 2020). Genomic prediction models have been trained using both historical phenotypic records and data from breeding programs (Gonzalez et al., 2021; Rembe et al., 2022). To manage the current influx of datasets from diverse fields, future tools taking advantage of methods from artificial intelligence, incorporating machine-learning and deep-learning methods/models (Alharbi and Rashid, 2022; Li et al., 2024c; Li et al., 2024d; Ma et al., 2024; Wang et al., 2023b; Yan et al., 2021), may provide a promising avenue to expedite future genetic gains (Farooq et al., 2024; Harfouche et al., 2019).

Breeding for hybrid cultivars through capturing heterosis

Harnessing hybrid vigor (or heterosis) is a major incentive to invest into establishing hybrid barley as a crop, despite barley's highly inbreeding natural mode of reproduction. Hybrid varieties of barley perform better in variable environments (e.g. lower fertilizer input, biotic/abiotic stresses conditions), and thus hybrid barley breeding is promising more stability in the face of global climate change (reviewed by (Fernandez-Calleja et al., 2022; Paril et al., 2024; Ramage, 1983)). The basic requirements of barley hybrid breeding are already met, and over 10 hybrid varieties have been commercially released to date in Europe from two breeding companies Syngenta and KWS. However, it's noteworthy that hybrid cultivars currently cover ca. 200,000 ha annually (Longin et al., 2012), accounting for only 0.42% of the global barley cultivation area. The future successful commercialization of barley hybrids will depend on identifying an economically significant level of heterosis, achieving sufficient cross-pollination and higher seed setting rate to make hybrid seed production economic and competitive, and developing an efficient and reliable system for the large-scale production of female parents and hybrid seeds.

Future hybrid barley breeding would benefit from the establishment and optimization of multiple hybridization systems. The first hybridization system in barley was the balanced tertiary trisomic (BTT) system, and it faded from the market a few decades ago (Ramage, 1983). This system included an extra translocation chromosome carrying a male fertile gene capable of restoring sterility caused by a recessive genic male sterile (GMS) gene, and led to the release of the first hybrid barley cultivar 'Hembar' in 1960s. The first commercial hybrid variety 'Colossus' was released in 2002 by New Farm Crops, Ltd, which has been integrated with Syngenta. It was produced using a cytoplasmic male sterility (CMS) system (HYVIRO® hybrid barley and sterile gene *msm1* + restorer gene *Rfm1*) (Rizzolatti et al., 2017; Ui et al., 2015), which requires a three-line approach involving a male sterile line, a nearly isogenic maintainer line, and a restorer line. The German breeding corporation KWS recently released its first hybrid 'Inys' (https://www.kws.com/gb/en/products/cereals/barley/variety-overview/inys/), a six-rowed feeding cultivar, relying on a system to sort yellow and blue aleurone grains which correspond to male sterile and maintainer lines, respectively. Both systems have been used for breeding six-rowed feeding barley varieties, with an ongoing demand for the balance of hybrid seed purity and production, both of which are relevant to seed pricing and competitiveness against elite inbreeds. The discovery of new sterility and restorer genes (e.g. in mutants and wild relatives) would enhance the CMS system to improve seed-setting and the flexibility of using restorer lines to hunt for great hybrid vigor. The environment-sensitive genic male sterility (EGMS) system is well-applied in hybrid rice seed production (Fan and Zhang, 2018), and examining GMS genes/mutants under diverse environmental conditions might lead to the identification of EGMS genes to develop a hybridization system in barley. The transgene-assisted GMS system has been developed in rice and maize, and a synthetic apomixis approach relying on the initiation of parthenogenesis via genome editing has also been proposed (Khanday et al., 2019; Song et al., 2024; Wang et al., 2019a). Both pioneering attempts might offer potential for establishing a cost-efficient hybridization system in barley, through following the same pipeline.

Once reliable hybridization systems are established, the next question is how to create hybrids that become economically significant in competition with elite inbred lines. This requires balancing the commercial interests of breeding companies and farmers. Scientifically, hybrid breeding will require simultaneous increases in both hybrid vigor and seed setting (cross-fertilization). The theory and pipelines to increase hybrid vigor in autogamous cereals, including barley, have been well reviewed (Longin et al., 2012; Melchinger and Gumber, 1998). Current studies on hybrid performance often failed to identify groups of genotypes with similar combining ability. The establishment of distinct heterosis groups in barley would benefit from the availability of extensive and genetically divergent barley germplasm stocks and increasing genomic datasets (Milner et al., 2019), as well as future transformation of hybridization systems in multiple backgrounds, followed by examining the performance of groups of hybrids. Breeding for elite hybrids would further require substantial increase of general combining ability through reciprocal recurrent selection and the selection of a higher better-parent heterosis. Targeted improvement of several traits necessary for cross-fertilization, such as the size and vigor of stigma and anther, degree of floral opening, anther extrusion, number of pollens per anther, and duration of pollen viability, as well as flowering time of male and female groups, is desirable. These floral traits can be identified through extensive investigation of barley mutants and germplasm resources, followed by gene isolation and marker-assisted introgression as previously mentioned (**Figure 3**). Uncovering functional mechanisms of floral-related genes and their applications in optimizing floral organs will enable the enhancement of these traits to increasing the seed setting rate applicable in large-scale hybrid seed production, expecting to result in the economic success of hybrid barley.

Barley as diploid model to explore hidden genes in common wheat

Barley is monophyletic and has only a third of the genome size of hexaploid wheat (Brassac and Blattner, 2015). The polyploidy nature of wheat might block the identification of recessive resistance, due to the functional redundancy of homoeologous genes that compensate for the genetic deficiency in any single gene. One such example is the recessive powdery mildew resistance locus *mlo* (Buschges et al., 1997; Wang et al., 2014). This problem of functional redundancy was also observed when recessive resistance genes sourced from *Ae. tauschii* were introduced into synthesized hexaploid wheat (Tang et al., 2023), with the synthetic hexaploidy being susceptible. The functional redundancy among the homologous genes in hexaploid wheat is more likely, due to the conserved macromolecular synteny observed across the three diploid genomes (**Figure 4A**). Furthermore, many genes exhibit phenotypic variation only when all three homoeoalleles are simultaneously edited (reviewed by (Zhou et al., 2023)).

The question is how can we recover the functionality of recessive genes that are blocked in hexaploid wheat? The advance of genome editing methodologies, such as CRISPR/Cas9 or base editing, presents an opportunity to efficiently investigate each or combinations of the three homoeoalleles (Awan et al., 2022; Li et al., 2021b). This approach necessitates prioritizing the identification of genes that are inherited recessively to ensure effective targeting and restoration of their function. Barley is genetically the closest major inbred crop to wheat (Bolot et al., 2009), exhibiting highest levels of genome collinearity and a high degree of sequence identity in high-confidence genes (**Figure 4A-B**). Both have been foundational crops in early human civilizations, and have dispersed globally together (Liu and Jones, 2024), adapting to rather similar environments. Barley stands as a globally cultivated crop with tremendous germplasm resources, representing an advantage over diploid wheat relatives such as *T. momococcum*, which is considered orphan, and *Ae. tauschii* that remains un-domesticated, respectively. The gene discovery in barley presents a strategic pathway for revealing recessive genes in hexaploid wheat. The strategy involves identifying recessive genes in the diploid barley, which then serves as a foundation for the targeted genome editing of their homologs in transformable wheat varieties (Abe et al., 2019; Kan et al., 2022; Kan et al., 2023; Wang et al., 2014) (**Figure 4C**), or marker-assisted stacking of non-transgenic mutagenized mutants (Acevedo-Garcia et al., 2017). This method promises to be a powerful tool in advancing our understanding and manipulation of genetic traits that are inherited recessively in hexaploid wheat.

CONCLUDING REMARKS

Breeding for barley varieties that exhibit improved yield performance, reduced resources input, and enhanced resilience to various abiotic and biotic stresses is a top priority for future barley breeding programs. Identifying the genetic loci, their corresponding genes, and functional regulatory elements that underlie agronomically important traits will facilitate the achievement of this ambitious goal. Current progress in barley pangenomes and functional genomics has opened new frontiers in barley research, offering valuable information to assist scientists and breeders for decision making in future pre-breeding (Hansson et al., 2018; Jayakodi et al., 2020; Jayakodi et al., 2024). However, we are still at the early stages of understanding how for instance large structural variations and functional DNA elements impact crop performance. The forthcoming generation of pangenome-scale sequence datasets from globally collected germplasm, along with in-depth analysis of phenotypes at target traits, coupled with extensive datasets of regulatory DNA elements and transcriptomes, will enable the linkage of agronomically important traits to specific genes, regulatory elements, and large structural variations. The optimal use of enriched mutant stocks (Hansson et al., 2024; Knudsen et al., 2022) and genotype-unspecific genome editing techniques (Wang et al., 2022), as well as multi-omics methodologies, will be crucial for in-depth unraveling genetic and functional mechanisms of important genes/traits, particularly those associated with environment-friendly agriculture. Collectively, the integration of genomic datasets, a rich array of germplasm resources, and the application of cutting-edge technologies, hold great promise for the future of barley research and breeding for sustainable agriculture in the coming decade.

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Figure Legend

Figure 1 Barley production, trade and innovation overview. (**A**) Barley grain production by country in 2022. This section illustrates the barley grain production across various countries, with those producing less than 1000 tons or having incomplete data represented in light grey. (**B**) Global barley production metrics since 1961. The graph displays the production, harvested area, and yield of barley grain on a global scale, with measurements in kilotons (kt) and kilohectares (kh). (**C**) End uses of barley grain from 2012 to 2021. 'Others' encompasses seed, food supply and loss categories. (**D**) Global barley trade dynamics since 1961. The export volumes, costs, and average prices per ton of barley in international trade are displayed. (**E**) Top-10 barley exporting and importing countries, based on the accumulation of quantity from 2013 to 2022. (**F**) Research and innovation in barley. The number of scholarly articles and patents with 'barley' as a keyword since 1912 is presented. The term 'articles' includes research and review articles. The data for these datasets were accessed on July 17, 2024, using FAOSTAT (https://www.fao.org/faostat/en/#data) and Web of Science (https://webofscience.clarivate.cn/wos/alldb/basic-search).

Figure 2 The architecture feature of a two-rowed barley plant at the 'milk' stage, along with the representative genes cloned through forward genetics approach referring various aspects of barley growing, developing and physiological performance. Details of these genes are given in **Supplemental Table 1**.

Figure 3 Strategy for mutant-based rapid gene isolation, allele mining, and marker-assisted introgression. (**A**) Forward gene mapping following the MutMap strategy. Mutants with desirable traits are crossed with the parental line used for mutagenesis. The resulting F_2 segregants are pooled based on the phenotype for whole genome sequencing (WGS). A candidate genomic region for the mutation is mapped by calculating allele frequencies in the two pools from WGS data, and mutations in candidate genes are detected using the same sequencing data. Once mutations at candidate genes are identified, phenotypic tests for the desirable trait are conducted to pinpoint the causal gene. (**B**) Reverse genetic approach for rapid gene isolation. Genes of interest within mutagenesis populations are genotyped using either genome-wide or gene-specific TILLING approaches. Mutants at the target genes are then screened for the trait of interest. FIND-IT, Fast Identification of Nucleotide variants by droplet DigITal PCR. (**C**) Mining for elite alleles. Germplasms are subjected to phenotyping and genotyping for the trait of interest. Association analysis is employed to identify elite alleles at specific traits. The polymorphisms among these alleles facilitate the development of molecular markers suitable for marker-assisted selection (MAS). (**D**) Marker-assisted introgression pipelines. The traditional pipeline, which relies on multiple rounds of MAS-assisted backcrossing, can be accelerated through the integration of speed breeding techniques that reduce the multiplication cycle duration. An alternative speed pipeline reduces backcrossing rounds and uses a larger population of BC_1F_2 segregants (Personal communication with Prof. Meixue Zhou, University of Tasmania, Australia). These plants are genotyped with genome-wide markers to select those containing the allele of interest while minimizing the genetic contribution from the donor line. The cross in the circle indicates selfing of F_1 plants.

Figure 4 Barley as a proposed diploid model for exploring recessive genes in hexaploid wheat. (**A**) Synteny analysis between barley H genome (Morex v3; (Mascher et al., 2021)) with the wheat ABD subgenomes (Chinese Spring v2.1; (Zhu et al., 2021)) was conducted using MUMmer v4.0.0 (Marcais et al., 2018) with minimum alignment length = 2 kb and minimum alignment identity = 90%. Visualizations were created using RectChr v1.38 (https://github.com/hewm2008/RectChr). (**B**) Identification of barley homologous genes in wheat was performed by analyzing the high-confidence (HC) genes of the barley Morex v3 reference genome and the wheat Chinese Spring v2.1 (Zhu et al., 2021), using the one-to-one module of GeneTribe software with default parameters (Chen et al., 2020). (**C**) Schematic diagram illustrating WYMV resistance through simultaneous knockout of three *TaPDIL5-1* homoealleles (Kan et al., 2022), the homologous gene of barley *HvPDIL5-1* (Yang et al., 2014). The black lines indicate the location of *PDIL5-1* gene on both barley and wheat genomes.

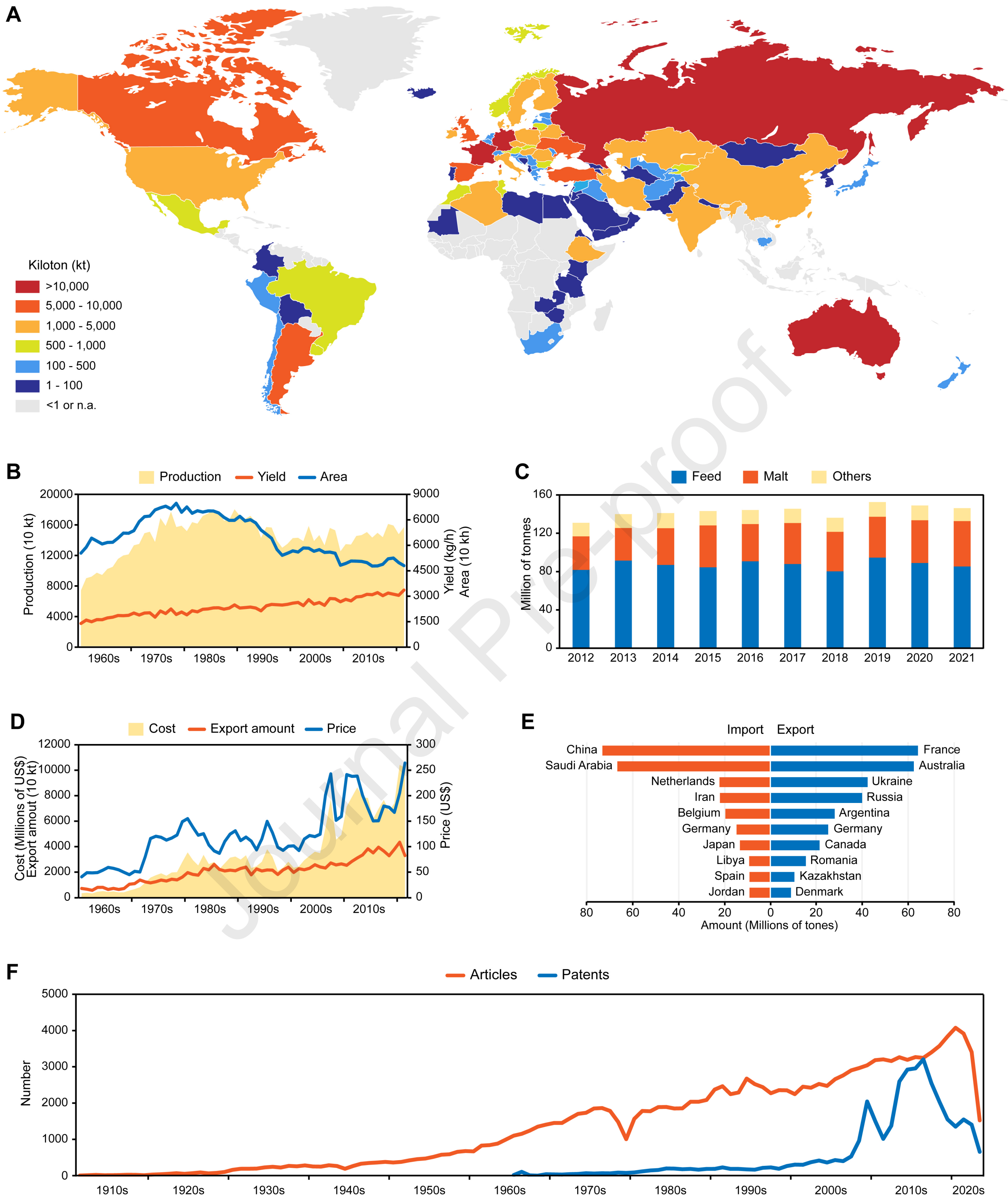
SUPPLEMENTAL INFORMATION

Supplemental Table 1 Barley genes that were cloned by forward genetic approach.

Supplemental Table 2 Databases offering search and analysis against barley genomic or phenomic data resources.

Supplemental Table 3 Major collections/populations representing barley natural diversity and induced variants.

Supplemental Table 4 Mutagenesis populations developed in barley over the past two decades.


A
Kiloton (kt)
>10,000
5,000 - 10,000
1,000 - 5,000
500 - 1,000
100 - 500
1 - 100
<1 or n.a.
B
Production
Yield
Area
Production (10 kt)
0
4000
8000
12000
16000
20000
Yield (kg/h)
Area (10 kh)
0
1500
3000
4500
6000
7500
9000
1960s
1970s
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Feed
Malt
Others
Million of tonnes
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120
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2012
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8000
10000
12000
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50
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1960s
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1990s
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China
Saudi Arabia
Netherlands
Iran
Belgium
Germany
Japan
Libya
Spain
Jordan
France
Australia
Ukraine
Russia
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Germany
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Romania
Kazakhstan
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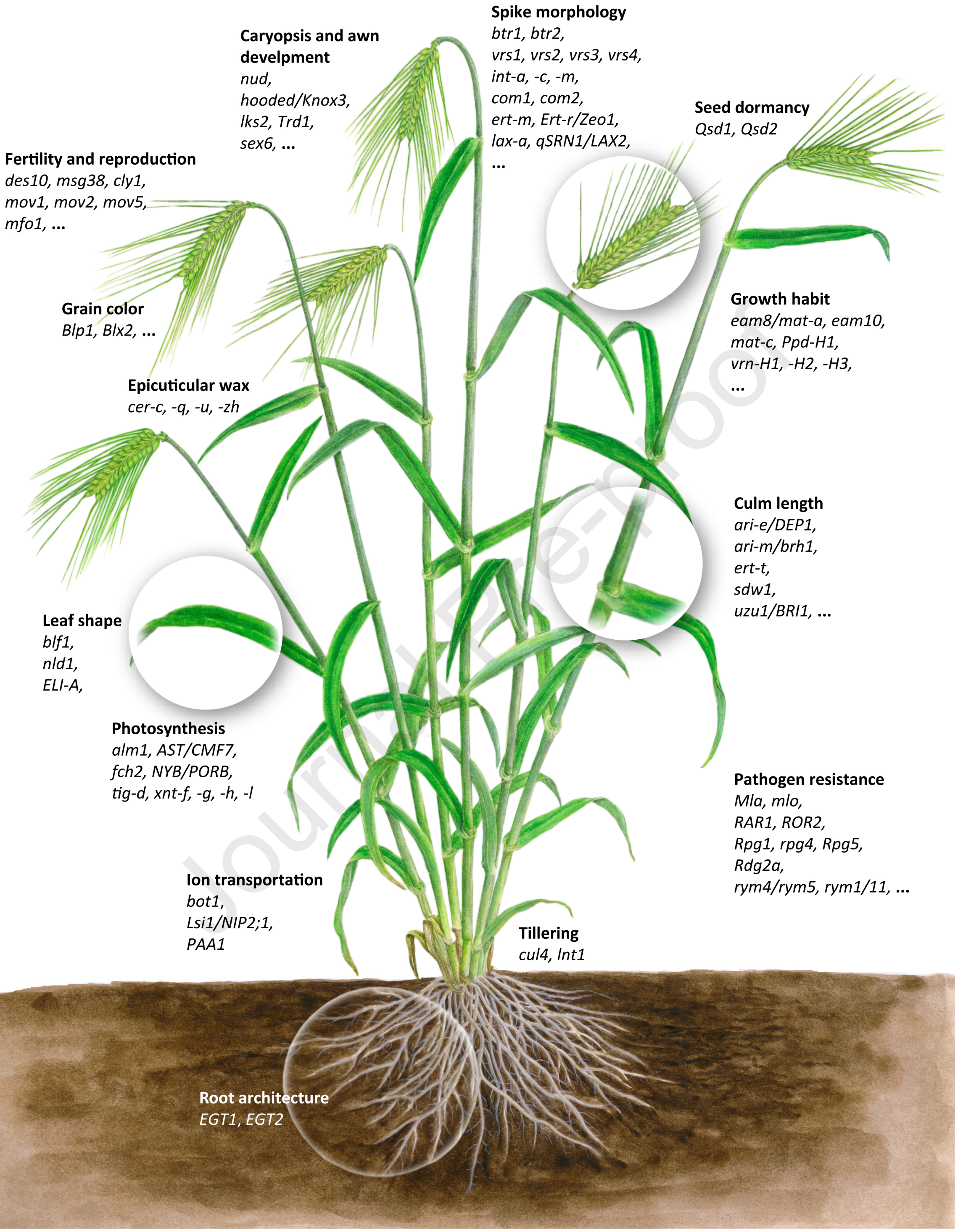
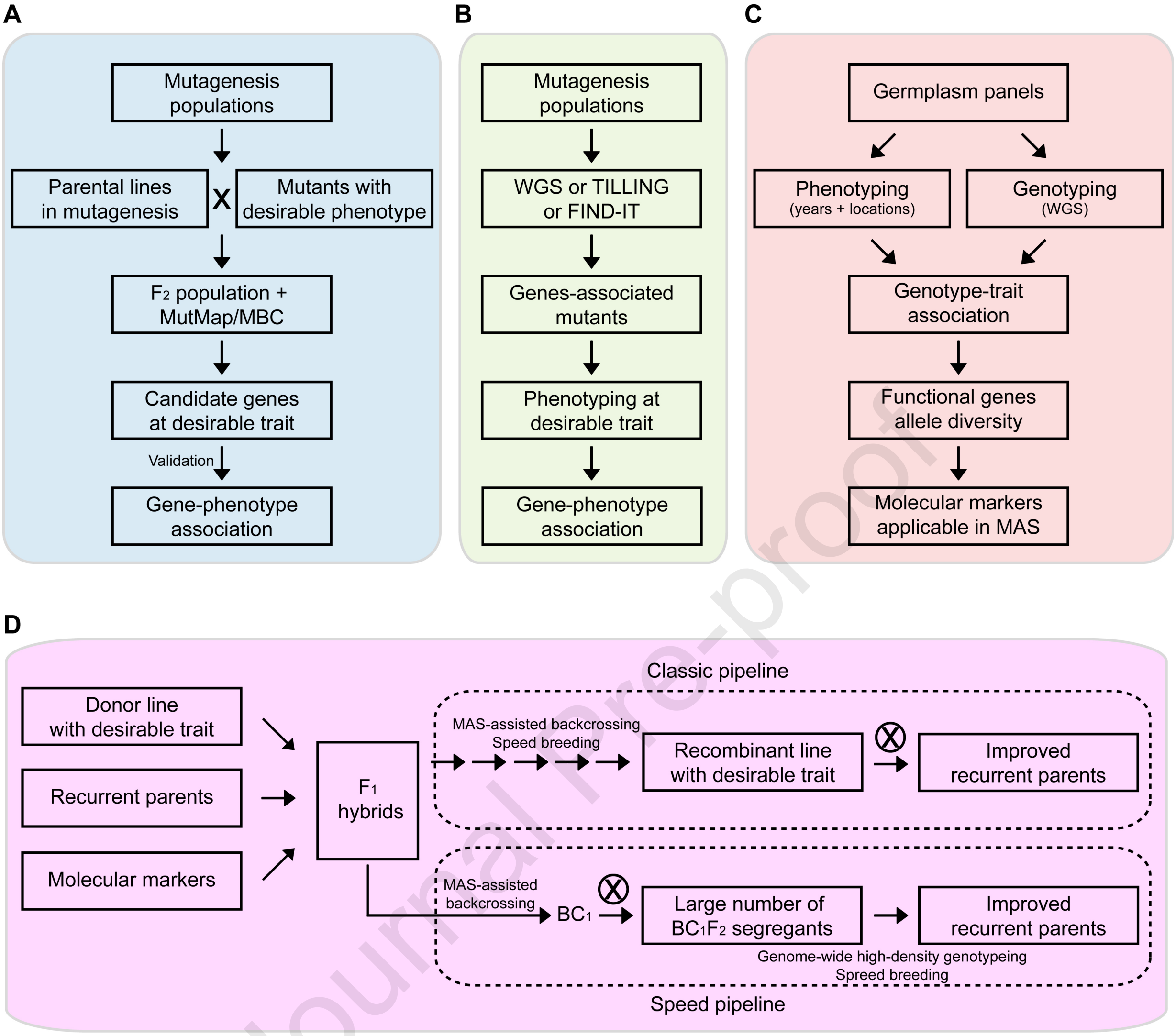
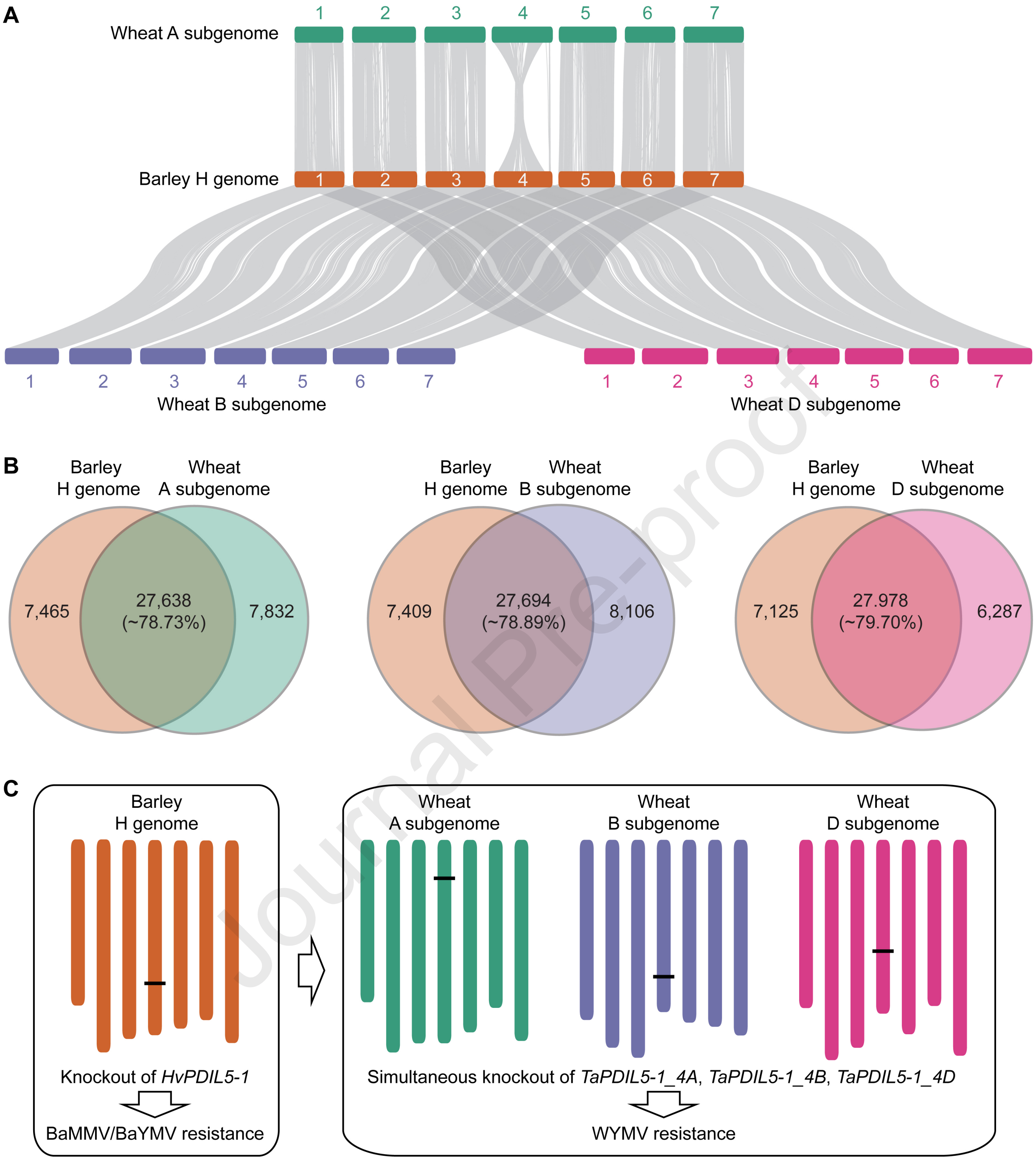

Spike morphology
btr1, btr2,
vrs1, vrs2, vrs3, vrs4,
int-a, -c, -m,
com1, com2,
ert-m, Ert-r/Zeo1,
lax-a, qSRN1/LAX2,
...
Caryopsis and awn develpment
nud,
hooded/Knox3,
lks2, Trd1,
sex6, ...
Seed dormancy
Qsd1, Qsd2
Fertility and reproduction
des10, msg38, cly1,
mov1, mov2, mov5,
mfo1, ...
Grain color
Blp1, Blx2, ...
Growth habit
eam8/mat-a, eam10,
mat-c, Ppd-H1,
vrn-H1, -H2, -H3,
...
Epicuticular wax
cer-c, -q, -u, -zh
Culm length
ari-e/DEP1,
ari-m/brh1,
ert-t,
sdw1,
uzu1/BRI1, ...
Leaf shape
blf1,
nld1,
ELI-A,
Photosynthesis
alm1, AST/CMF7,
fch2, NYB/PORB,
tig-d, xnt-f, -g, -h, -l
Pathogen resistance
Mla, mlo,
RAR1, ROR2,
Rpg1, rpg4, Rpg5,
Rdg2a,
rym4/rym5, rym1/11, ...
Ion transportation
bot1,
Lsi1/NIP2;1,
PAA1
Tillering
cul4, lnt1
Root architecture
EGT1, EGT2


A
Mutagenesis populations
Parental lines in mutagenesis
X
Mutants with desirable phenotype
F2 population + MutMap/MBC
Candidate genes at desirable trait
Validation
Gene-phenotype association
B
Mutagenesis populations
WGS or TILLING or FIND-IT
Genes-associated mutants
Phenotyping at desirable trait
Gene-phenotype association
C
Germplasm panels
Phenotyping (years + locations)
Genotyping (WGS)
Genotype-trait association
Functional genes allele diversity
Molecular markers applicable in MAS
D
Donor line with desirable trait
Recurrent parents
Molecular markers
F1 hybrids
Classic pipeline
MAS-assisted backcrossing
Speed breeding
Recombinant line with desirable trait
⊗
Improved recurrent parents
MAS-assisted backcrossing
BC1
⊗
Large number of BC1F2 segregants
Improved recurrent parents
Genome-wide high-density genotypeing
Spreed breeding
Speed pipeline


A
Wheat A subgenome
1
2
3
4
5
6
7
Barley H genome
1
2
3
4
5
6
7
1
2
3
4
5
6
7
Wheat B subgenome
1
2
3
4
5
6
7
Wheat D subgenome
B
Barley H genome
Wheat A subgenome
7,465
27,638 (~78.73%)
7,832
Barley H genome
Wheat B subgenome
7,409
27,694 (~78.89%)
8,106
Barley H genome
Wheat D subgenome
7,125
27.978 (~79.70%)
6,287
C
Barley H genome
Knockout of *HvPDIL5-1*
BaMMV/BaYMV resistance
Wheat A subgenome
Wheat B subgenome
Wheat D subgenome
Simultaneous knockout of *TaPDIL5-1_4A*, *TaPDIL5-1_4B*, *TaPDIL5-1_4D*
WYMV resistance