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GWAS: Fast-forwarding gene identification and characterization in temperate Cereals: lessons from Barley – A review

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GWAS: Fast-forwarding gene identification and characterization in temperate Cereals: lessons from Barley – A review



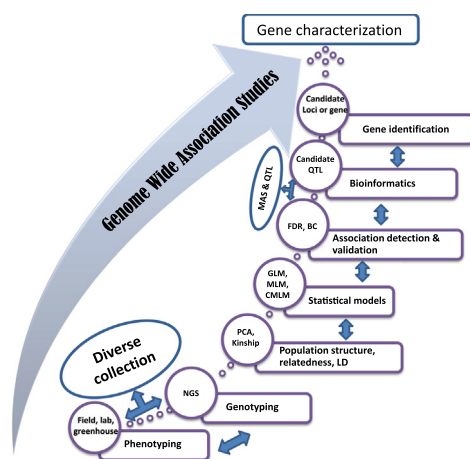
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GRAPHICAL ABSTRACT



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ABSTRACT

Understanding the genetic complexity of traits is an important objective of small grain temperate cereals yield and adaptation improvements. Bi-parental quantitative trait loci (QTL) linkage mapping is a powerful method to identify genetic regions that co-segregate in the trait of interest within the research population. However, recently, association or linkage disequilibrium (LD) mapping using a genome-wide association study (GWAS) became an approach for unraveling the molecular genetic basis underlying the natural phenotypic variation. Many causative allele(s)/loci have been identified using the power of this approach which had not been detected in QTL mapping populations. In barley (*Hordeum vulgare* L.), GWAS has been successfully applied to define the causative allele(s)/loci which can be used in the breeding crop for adaptation and yield improvement. This promising approach represents a tremendous step forward in genetic analysis and undoubtedly proved it is a valuable tool in the identification of candidate genes. In this review, we describe the recently used approach for genetic analyses (linkage mapping or association mapping), and then provide the basic genetic and statistical concepts of GWAS, and

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subsequently highlight the genetic discoveries using GWAS. The review explained how the candidate gene(s) can be detected using state-of-art bioinformatic tools.

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Introduction

Natural variation is a valuable and sustainable resource of the phenotypic and genetic diversity within plant species (e.g. barley, *Hordeum vulgare* L.) worldwide that offer beneficial traits for plant breeding. The phenotypic variation within-species caused by spontaneously natural genetic mutations that maintained in nature by evolutionary, artificial and natural selection processes [1]. Natural variation brought great advances to understand crop morphology and their response to biotic and abiotic stresses. The understanding of natural variation in crop plants through thousands of years for domestication e.g. in barley about 10,000 years ago [2] can be seen in the genetic modification of developmental traits and adaptive features. Natural variation studies in wild species elucidated the molecular basis of phenotypic differences related to domesticated plant adaptation that is important to interpret the maintenance and evolutionary significance of phenotypic variation [3]. For instance, *Six-rowed 1* (*VRS1*) and *Non-brittle rachis 1* (*btr1*) or *Non-brittle rachis 2* (*btr2*) genes in barley have clear impact on spike architecture phenotype as a consequence of domestication [4,5]. During domestication, loss of function in *VRS1* gene converted the two-rowed barley to six-rowed that increased the grain number per spike and the deletions in *Btr* genes make non-brittle rachis that improved grain retention. Analyses of natural variation within wild and/or domesticated, cultivated plants diversity can help to utilize the diverse resources for crop improvement efficiently and improve the knowledge of the genetic basis of cultivated crop improvement. Genetic analyses of natural quantitative variation in crop plants were developed a few decades ago [1]. A genebank provides a rich source of genetic variation that had been greatly used to improve cultivars through incorporating the desired alleles into breeding programs for increasing grain yield and improving tolerance to abiotic and biotic stresses [6]. The genetic bottlenecks that happened during domestication and modern breeding processes lead to a narrowing of the genetic variation in cultivars that negatively affects productivity, adaptation and yield sustainability [6].

Barley is grown in areas where other close relative cereal crops like wheat (*Triticum spp.*) are poorly adapted and it is now cultivated in all temperate regions of the world. Therefore, barley became a basic crop for human civilization and approximately 70% of production is used for animal feed, 20–25% malting, and 5–10% for food [7]. Presently, it is ranked as the fourth most important cereal crop in the world [8] while Europe and the Russian Federation produce 65% of global production. Being related to wheat and with it is economic and agronomic importance, numerous genetic and genomics studies in barley have been used as a model crop for wheat. Barley's research has dramatically expanded in the last few decades with more than 25,000 publications since 1980 based on Web of Science™. Barley has many features of a model species. Since barley has over 400,000 accessions in gene banks [9], it offers an excellent resource to efficiently exploit genetic resources and their utility for breeding programs. In combination with a new ordered high-quality reference genome sequence assembly [10], barley became a crop with a much more tractable genome. Consequently, barley remains an important model plant that can be used to understand the genetic basis of adaptation to various abiotic and biotic stresses (predicted with climate change).

The recent advances in DNA sequencing paved the way to genetically improve the important traits (grain quality, biotic and biotic stress tolerance, etc). Next-generation sequencing (NGS) e.g. genotyping-by-sequencing (GBS) provide thousands of single nucleotide polymorphism (SNPs) covering the most genomic region in barley chromosomes. Many powerful statistical genetics methods were proposed to identify alleles controlling target traits. Genome-wide association study (GWAS) is one of those useful methods and it is successfully used to identify candidate genes for many important traits in barley as it tests the association between the marker type (e.g SNP) and the phenotype of a target trait. There are many considerations and recommendations that should be taken into account when geneticists decide to perform GWAS. In the current review, we will discuss the advantages and distances of GWAS, different methods for performing GWAS, and a brief guide of interpreting GWAS results. We focused on barley studies in our review as a excellent example of a crop that has a significant genetic improvement due to the identification of many useful QTLs and genes, that were used in marker-assisted selection, using GWAS.

Genetic studies of complex traits

Forward genetics aims to screen the phenotype of many individuals that are genotypically different. Understanding the relationship between genetic polymorphism and the phenotypic variation observed among individuals is one of the fundamental interests. This basic relationship has been extensively studied since Mendel demonstrated that this relationship is inherited. Revealing the genetic factors underlying complex characters such as agronomically important traits like grain yield requires an understanding of allelic variation at a specific locus level that controls the phenotype and the genetic architecture of a given trait. The variation of the plant phenotype is directly connected back to the underlying causative loci using mapping approaches. To achieve this goal, phenotypic and genotypic differences among the individuals are studied either using bi-parental QTL mapping populations (linkage mapping) or association mapping populations (LD mapping) of unrelated individuals. Therefore, both mapping approaches aim to identify molecular markers that are linked to QTL.

These approaches became attractive and useful because they utilize the advances in genome sequencing and high-quality and density SNP arrays for many crops, including barley. Recently through NGS, most of the populations are genotyped by either a 9 K iSelect Illumina Infinium array [11] that contains 7,842 gene-based SNPs of which 6,094 SNPs have known physical positions (the location of identifiable landmarks of SNP on the chromosome which always measured in base pairs), or a 50 k Illumina Infinium iSelect genotyping array contains 44,040 SNPs which represent 29,415 unique gene pseudomolecules annotations [12]. The 50 K array increased the density of the high-quality markers i.e. a higher number of SNPs (14,626 SNPs) compared with 4,570 SNPs in 9 K [13]. The NGS offers an effective and relatively low-cost approach to rapidly map the population using GBS. The GBS technique uses restriction enzymes to reduce the complexity of DNA samples and then produce high-quality polymorphism data [14]. Early use of GBS found 1,596 SNPs [15], hence the 50 K chip provided better coverage of the genome. Even though genotyping using SNP is

Table 1
The main advantages and limitations of QTL mapping and GWAS mapping approach.

QTL mapping		GWAS mapping	
Advantages	limitations	Advantages	limitations
Bi-parental crosses	Contrasting and crossable parents and multiple generations required to develop pedigrees	No parents or crossing	Population structure effect with spurious relatedness
Fewer markers required	A limited number of genotypes based on the success of crossing Low allele richness	Unlimited number of contrasting accessions Assumes dense markers with high allele richness	The high number of individuals are required Low allele frequency
Expecting the segregating trait(s)	Lower resolution based upon the number of recombination	Large numbers of phenotypic variation	Misleading natural variation
More robust in heterogeneity	Narrower genetic base	Highly dense map	Low heritability value
Less prone to false positives	Markers are usually sparse due to the recombination	Higher resolution and tests at marker positions	Many more markers required
Tests between markers			Type I or II error (false positive association)

extremely efficient and reliable, the GWAS performed over the past decade explored some drawbacks that should be considered. GWAS based on SNP relies on the pre-existing genetic variant reference that is used for sequencing and mapping the individuals. Such specific design leads to missing pinpoint causal variants and cannot detect most of the genetic signals or rare mutations of complex traits. Based on the Web of Science™ database, since 1991 around 1300 QTL studies using parental populations are listed compared to only 90 GWAS publications in barley.

In the next sections, QTL mapping and GWAS methods will be discussed with more focus on the GWAS method.

QTL mapping (linkage mapping)

The linkage or QTL mapping approach is commonly used to identify genomic regions (QTL) controlling target traits. The family-based mapping analysis depends upon the genetic recombination and segregation during the construction of mapping populations in the progenies of bi-parental crosses that consequently affect the genetic mapping resolution and allele richness. QTL mapping has proved and remains a powerful approach to identify loci that co-segregate with the trait of interest in the research population. This approach can be applied in different types of populations e.g. F₂ populations, double-haploid (DH) populations, backcross, or recombinant inbred lines (RILs) families, using restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR), and SNP markers.

QTL analysis has been widely applied in barley for the genetic dissection of agronomic traits using genetic maps constructed from RFLP markers [16,17] or applying other genetic maps using e.g. SSRs [18]. QTL analysis for developmental and yield traits was carried out in DH and RIL mapping population using AFLP [19] while SSR markers were used to detect QTL for physiological, biochemical, agronomic and yield traits in RIL population [20,21]. Recently, SNP chip started to be used in QTL studies e.g. Huang et al. [22] used them in a RIL population to discover QTL for agronomic traits and fusarium head blight. GBS is also used for QTL analysis in RIL that allowed them the detection of the *Breviaristatum-e* (*ari-e*) locus [15].

Multiple environment trials (i.e. locations and/or years) for studying complex traits are commonly used to assess the performance of genotypes across a range of environments, including QTL × environment interaction (QEI) to find important and broadly adapted QTL. There are several studies focused on agronomic traits such as heading date, thousand-grain weight (TGW) and plant height in barley using this approach e.g. [23,24]. Many QTL studies have been carried out to study the genetic factors underlying

drought-related traits in barley, revealing that most of the detected QTL control developmental and adaptive traits in addition to drought tolerance. Rollins et al. [25] detected numerous QTL under dryland conditions using SSR and diversity arrays technology (DART)-markers for constructing a genetic linkage map in the RIL population. Using such a combination of markers, QTL analysis demonstrated that heading date related-genes (in particular the vernalization genes *Vrn-H1* and *Vrn-H2*) had pleiotropic effects on yield-related traits and biomass.

The major fundamental limitations in QTL mapping are that the diversity of segregating alleles between the parents can be only tested, and the mapping resolution solely relies on the number of recombination events that occurs during the population development [26]. Developing pure lines (homozygous lines) for mapping populations is time-consuming and results in a low resolution of mapped QTL as an outcome of a low number of recombinations caused by the few numbers of genotypes resulting in a narrow genetic base with low allele richness (Table 1). Through conventional breeding, six to eight generations of introgressions or selfing are needed to form pure lines (homozygous lines) of RILs or near-isogenic lines (NILs) populations while two generations to form a DH population with a lower chance of recombination rate events than RIL population [27]. This may be due to the fact that DH lines only go through one round of recombination while RILs, on the other hand, go through many rounds of recombination. Homozygous lines can be also produced from F₂ using a single seed descent method where one seed is harvested from each F₂ line and then grown into an F₃ and so on until F₈ to F₁₀ generations with high levels of homozygosity at virtually all loci. Finally, the members of a family-based mapping population will contain different amounts of recombination among loci.

To avoid these limitations, improvement in the mapping resolution within the mapping population can be dramatically improved by increasing the number of intercrosses using multiparent RILs [28]. There are many positive features in using this approach. It requires high-density markers in case of a high recombination rate (RIL lines) for mapping QTL and to identify tightly linked markers. It is also robust to understand the heterogeneity at the locus level. Advanced molecular technologies e.g. GBS allowed for rapid and cost-effective genotyping (hundred to thousands of markers) with high allele richness that make QTL mapping robust and useful for identifying the target region of complex traits.

Genome-wide association study (GWAS)

Association analysis using GWAS is a powerful tool being effectively and efficiently used for genome-phenotype associations and causative loci/genes identification. The basic scenario in GWAS is

to calculate the association between each marker and a phenotype of interest that has been scored across unrelated lines/individuals (unrelated individuals means distantly related and heterogeneous individuals) of a diverse collection [29]. Robustness and effectiveness of GWAS in the dissection of complex traits in crops including barley had been demonstrated and expected to become more efficient to identify the causative loci/gene(s) for quantitative traits with a help of the currently available large populations and high-throughput sequencing technology. High-resolution mapping can also be attributed to historical recombination events [30] and the greater allele numbers that are incorporated in GWAS. In the association mapping populations, historical recombinations that accumulated over generations with historical Linkage Disequilibrium (LD, over dozens/hundreds of generations) persist among the representative accessions and improved the resolution for association analysis through the rapid decay of LD. Unlike association mapping populations, family-based populations, particularly DH populations, having a limited number of recombination events will often generate populations with relatively low mapping resolution and wide recombination value for a pair of loci, hence a larger linkage block that increases LD.

The application of sophisticated analytical approaches has started to extend the utility of different genetic resources for studying the natural variation that can be ultimately used in improving the crop. This approach has been studied extensively in humans and also started in plants since the beginning of this century [31]. Early reports used this approach in plants were on diverse maize (*Zea mays* L.) population [32] and *Arabidopsis* [33], thereafter the approach was used in other crops and the number of published reports increased, see the review by Rafalski [30]. In barley, this approach started ten-years ago [34,35].

Recently, GWAS has become a key approach for mapping quantitative traits and studying the natural variation. GWAS with high-density genotyping platforms provides enough marker density to dissect the genetic architecture of traits of interest in barley. Through screening large and diverse collections with ample genetic marker density, GWAS can detect causal loci underlying natural phenotypic variation. For instance, GWAS analysis using a 9 k SNPs chip from IlluminaTM [11], a gene-based chip providing a high genetic resolution that can help to uncover novel alleles that improve productivity and adaptation. In barley, the GWAS approach will become more robust and informative using the newly developed 50 k Illumina Infinium iSelect genotyping array for barley [12].

Natural variation of phase transition especially heading date is one of the critical traits that are highly associated with adaptation and yield [36]. Understanding the natural variation of heading date is important to increase our knowledge regarding the natural diversity of other developmental traits such as leaf area, plant height, tillering, grain number, or other agronomic traits [37–42].

The main objectives of performing GWAS are to identify causative factors for a given trait and/or to determine the genetic architecture of the trait. The number of loci underlying the phenotypic variation of traits differs i.e. the trait can have a simple genetic architecture with a low number of large-effect loci (e.g. barley spot blotch) or a complex genetic architecture and controlled by many loci (polygenetic e.g. heading date) [43].

Important factors affecting the power of GWAS

The power of GWAS to detect the true association is determined by many factors which should be taken into account when geneticists and breeders perform GWAS for target traits, Table 1 summarized the advantages and limitation of GWAS which are described as follow:

First: phenotypic variation. The raw phenotypic data should be filtered from the outliers which are noisy data points for further analysis. Keeping these points can shift the phenotypic data from a normal distribution which is considered as a limitation of GWAS that can later affect the natural diversity analysis. The simple way to know how many outliers are in the phenotypic data and whether they are effective or not is to use a boxplot that can easily visualize the data and extreme outliers should be excluded. Meanwhile, the phenotypic variation is an important part of the association analysis and removing outliers should not affect it in a meaningful manner. Moreover, traits only with moderate to high heritability estimates (for the phenotypic data after filtration) should be considered in GWAS because heritability is a good indicator of how much the genetic variance contributed to the phenotype and how much the phenotype is linked to the genotype. Low broad-sense heritability is a limiting factor that reduced the power of GWAS to detect the association. Genotypes repeated across locations or years may have a strong genotype \times environment interaction which reduces the heritability of a trait. There are many methods such as best linear unbiased predictor (BLUP) and best linear unbiased estimator (BLUE) that can be used to adjust the phenotypic data scored across locations or years to provide better estimates of the phenotypic values considering genotype \times environment interaction. The relationship between the associated SNP and phenotypic traits in unrelated individuals is explained by the estimation of the variance of SNPs which when used in a GWAS is also known as the so-called SNP-based heritability. Such analysis helps in dissecting genetic variation and understanding the genetic architecture for complex traits, in addition to identifying the most significant SNP that can be incorporated in future breeding programs.

Second: the number of individuals. The population size is very important for obtaining meaningful results. Population size is critical to define portions of the phenotypic and genotypic variation; hence increasing the population size will improve the power of having meaningful associations with a larger effect, an acceptable frequency within the population, and overcome rare-variants. Thus, a low number of individuals is a disadvantage that reducing the power of GWAS. A range of 100–500 individuals are needed and suitable for performing GWAS [44]. The individuals of the population may be selected based upon their expected phenotypic and genotypic variation considering genetic background, including geographic regions, biological status, growth habit or whatever trait the researchers interested in. The selected individuals should be replicated to confirm their diversity through statistical analyses including clustering analyses and to ensure a normal distribution. In case the individuals do not have extensive genotypic information, it is possible to estimate their genetic diversity using a few molecular markers for some important genes e.g. photoperiod response, vernalization response, plant height, and row-type. After a phenotypic and genetic analysis, we keep those individuals which show high variation in the population. Finally, sufficient seed for further research purposes is needed. The population should be grown and isolated (selfing) for at least one growing season with a preference for multiple selfing generations for multiplication and purity e.g. single seed descent. Careful selection of population individuals can have large genetic variation and detect true novel association signals that can be used for further breeding and genetics aspects. Most of barley GWAS studies used hundreds of individuals which were selected to represent different geographical regions, growth habits, row-types, etc. which maximize the genetic variance to detect the specific allele(s). This approach also increased the chance for genetic heterogeneity within the population that may reduce the power of GWAS to detect major loci, leading to a non-causative allele(s), and affect the allele estimates of the marker. A high number of samples with low genetic

heterogeneity (from the same region, growth habit or row-type. . .) may not show expected phenotypic/genotypic diversity or the allelic variation present at low allele frequency or absent completely. Therefore, a high number of globally diverse individuals would be the best solution to make the balance between genetic diversity and allele frequency e.g. GWAS for highly heritable and routinely scored morphological traits during genebank propagation (e.g. row-type, hull adherence and awn roughness) in a 1,000-sample core set from 21,405 accessions of the IPK barley collection using GBS [45]. Many studies on wheat dealt with the problem of heterozygous loci in the association panel by considering all heterozygous loci as missing and re-filtering the marker data [46,47] and similarly was done in faba bean, *Vicia faba* L [48].

Third: population structure. It is a statistical approach that aims to calculate relatedness correlation among individuals within the population due to the admixture and historical structure that must be considered carefully during the analyses and results interpretation. The selection of a population for association analysis by researchers generates the structure, based on geographic or growth habit, etc. that leads to having a specific genetic variation and an effect on the end-use of association analysis. It is the major limitation in the GWAS analysis since not all individuals are equally distantly related to each other at the genetic level. Ignoring the correction of population structure leads to having spurious associations between genotype and the trait of interest. The STRUCTURE program is a computationally intensive method to define the population structure and then estimate the proportion of clusters (unknown number of subpopulations) within the population so-called Q matrix and then estimates which individual belongs to which subpopulation [49]. The software produces highly accurate clustering using multilocus data from the genotypes to explain the population structure. Removing structured associations is not always adequate for controlling the population structure due to the limitation in defining the number of clusters and how to assign individuals into clusters. In addition, structure analysis can be

time-consuming requiring intensive computational analysis. Alternatively, the EIGENSTRAT method using principal component analysis (PCA) is another statistical approach developed by Price et al. [50] that counts the structure of the population in order to reduce the dimensional genotype data to control the structure. The method considers the genotypic data to deduce genetic variation that can be explained by a small number of dimensions. Yu et al. [51] developed a mixed-model approach to control spurious associations through accounting multiple levels of relatedness through a pairwise relatedness matrix called the kinship matrix (K). K can calculate the relatedness between pairs of individuals using genotypic information. The high value of relationships among the individuals indicates high genetic similarity e.g. the tendency among the individuals from the same geographical region which can be clustered in the group. Most of the studies use both methods (STRUCTURE and PCA) to confirm their results [38,45,52,53]. The principal component analysis is presented in a scatter plot of PCA1 and PCA2 which present the most of the total variation between the individuals based on their genotypic data. If the genotypes are randomly distributed in the plot and form no clear groups, then there is no population structure in the population, and *vice versa* (Fig. 1a and b). In STRUCTURE software, the population structure is determined by plotting the proposed number of subpopulations against delta k [54]. However, if the number of subpopulations is assigned into two subpopulations, the population could have two possible subpopulations or no population structure because STRUCTURE does not estimate delta k for the first subpopulation. The presence or absence of population structure can be determined by another plot in which a number of subpopulations are plotted against log-likelihood. In the case of no population structure (Fig. 1c), the log-likelihood is steadily increased with the increase of a number of subpopulations. If the log-likelihood, on the other hand, is steadily increased after $k = 2$ (Fig. 1d), then this population can be divided into two possible subpopulations. Structure Harvester (<http://taylor0.biology.ucla.edu/structureHarvester/>)

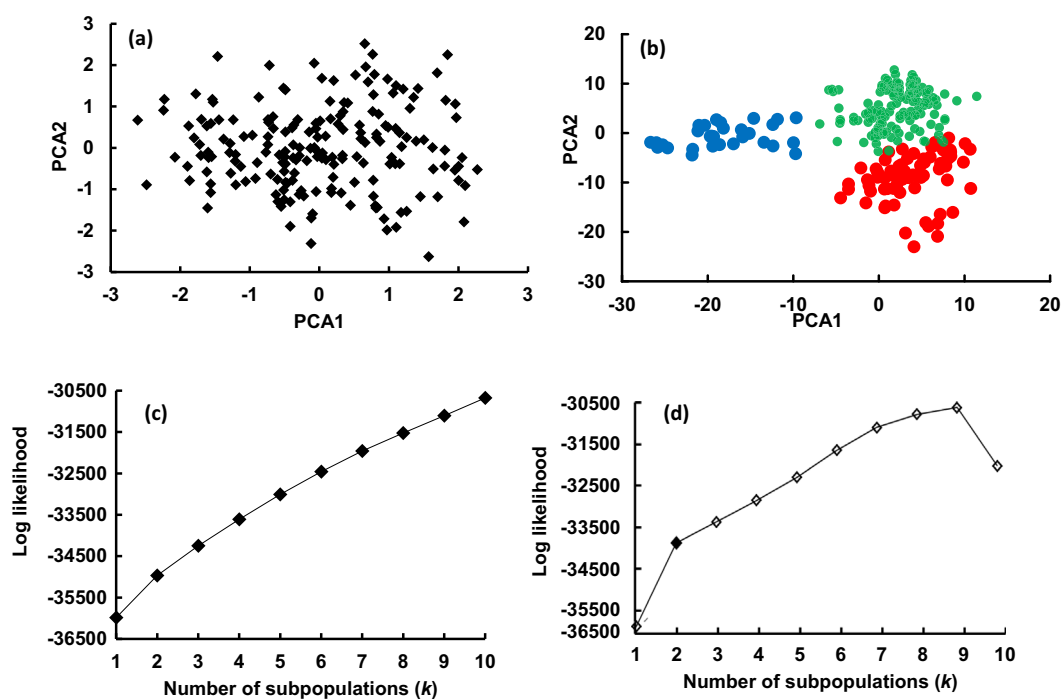


Fig. 1. Visualization of population structure and number of subpopulations within the population. No clear population structure (a), whereas the population was well-structured (b). Log probability data as function of k (number of clusters/subpopulations) from the STRUCTURE run. No number of subpopulations (c), while two subpopulations are shown in (d). Each color in (a) and (b) represents a subgroup and each dot represents an accession/individual. PCA, principal component analysis.

is a very useful website in which the output results of STRUCTURE can be compressed and uploaded. The software provided information on population and the best k for the proposed populations in table and figures.

Fourth: allele frequency. A very important factor, which affects the power of GWAS, is if alleles are present in a few individuals across the population. Rare allele leads to a lack of resolution power [55]. Therefore, allele frequency distribution and analysis impact on detecting the association. It is difficult to detect the functional alleles that are present at a low frequency unless they have high impacts on the phenotype. Ignoring the allele frequency might mislead the GWAS outputs. Most of the GWAS studies focused solely on common variants and have the major allele frequency at $\geq 5\%$. This approach means that in the population of 200 individuals, the allele present in 10 individuals or less will not be detected because it is a rare variant with minor allele frequency (MAF) at less than 5%. Unfortunately, rare alleles could explain natural variation in a specific group of individuals that is important for further breeding and genetics in addition to biological studies. For example, deep analysis of GWAS revealed that a group of East Asian accessions (13 accessions out of 209) is carrying the allele (MAF ≥ 5 , i.e. the allele present in 11 accessions) that led to significantly longer phase durations, lower tiller numbers, more leaves, and a greater leaf area [56]. This finding confirmed that low-frequency alleles may have relatively large effects on complex traits and suggested that the population structure should be well studied and connected to the GWAS output to interpret the findings. It is also important to mention that the selection process of individuals has a clear impact on variants across the allele frequency that can be skewed for traits which are strongly influenced by selection. In most crops, the domestication bottleneck has a clear impact on the allele frequency by eliminating many rare alleles and reduced the average allele frequencies. Therefore, careful selection of a large number of representative individuals (including wild relatives and landraces) with advances in genotyping (e.g. GBS) will further boost the power of GWAS to detect the MAF by increasing the number of SNPs. Furthermore, a deep analysis of the association signals including the flanking SNPs and group-specific individuals (e.g. wild and landraces) could, on one hand, increase the power of discovering variants with a strong effect on the phenotype. Alternatively, it could potentially detect the causative association signal. Overall, discovering the highly informative rare alleles will provide more powerful genetic tools to answer biological questions.

The linkage mapping population is a good choice for dealing with rare alleles since it can be artificially introduced. Several studies have used linkage mapping along with LD mapping, which resulted in a methodology known as “nested association mapping” which decreases spurious associations by considering the population structure. Recently, Nested Association Mapping (NAM) has been developed in barley to investigate the genetic architecture of complex traits using GWAS. Halle Exotic Barley 25 (HEB-25) is a multi-parental mapping design to use the advantages of linkage analysis and association mapping by crossing 25 wild barley (*H. vulgare ssp. spontaneum*, *Hsp*, and *ssp. agriocrithon*, *Hag*) accessions with the spring barley cultivar Barke (*H. v. ssp. vulgare*, *Hv*) which offered an exceptional genetic resource [57]. Allelic diversity within the mapping population can be increased through intercrossing multiple parents which are genetically diverse e.g. the Multi-parent Advanced Generation Inter-Cross (MAGIC) [58]. Another issue that should be considered is allelic heterogeneity at a single locus level (multiple alleles/genomic markers might have similar effects on the trait of interest), or at loci heterogeneity if the heterogeneity occurs in several distinct genes [59]. Incorporating multiple neighboring markers to the strongest associated signal is the solution in small grain cereal crops e.g. barley and wheat because we still work with a few thousands of SNPs.

Fifth: LD is another point that has to be considered during the analyses, especially to define the interval of highly associated SNPs that can lead to defining the most significant loci. Ignoring the non-random association among alleles at different loci means that both causal and non-causal alleles will be incorporated in the further analyses that likely driving to have false associations. The LD is an indicator to detect the distance between loci, which is important to find the number of required markers for the whole-genome scan, i.e. high LD value means a low number of markers are needed to cover the genome [60,61]. A long-range LD increases the chance of false association and therefore, the calculation of LD at the beginning of the association analysis is essential. The coefficient of LD is used to measure the value of how likely two loci are associated and sharing the history of mutation and recombination. This analysis always includes a disequilibrium matrix that shows the pairwise calculations among loci using the most two common statistics for measuring LD i.e. r^2 and D' [31]. According to many LD analyses in plants, r^2 is a stronger value to estimate how loci correlate with the QTL of interest while D' is more affected by small population sizes and low allele frequencies. Because LD is used to calculate the association value between loci (r^2 or D' , >0) it is important to connect the phenotypic variation with the causative SNPs. LD between SNPs, including the causative locus (within LD) must be considered in a statistical analysis that can show whether each SNP within the LD is significantly associated with the phenotypic variation or not. Here, we propose to consider all SNPs above the threshold (in some cases all SNPs) in such analysis to check which one can explain more natural phenotypic variation since it is known that not all of the highly associated SNPs having a highly significant impact on the phenotype. The SNPs which are in LD with $r^2 > 0.2$ should be considered in the statistical analysis which can be useful to detect the causative loci especially for QTL that are located in the centromere region. Another feature of calculated r^2 as an estimate of LD between each pair of SNPs is that it gives important information if a group of significant SNPs tends to be inherited together or representing the same QTL or individual QTLs. By looking on the significant SNPs located on the same chromosome, if the r^2 value between the two SNPs is high, then these SNPs probably represent the same QTL and tend to be inherited together, while, if the value is low, then the two significant SNPs probably represent two different QTLs.

To clarify, LD is an estimate of map distance with the consideration of allele frequencies, whereas linkage refers to chromosome-level [31].

The map resolution of a given population (i.e. the number of markers and density) is determined by genome size and LD decay (the rate at which LD declines with genetic or physical distance). How quickly LD decays over distance (genetic/physical) has been shown to differ dramatically and vary significantly among species, within the genome and for loci within a population. The rapid decay of LD requires a large number of markers to be used in the whole-genome association analysis [62]. The rate of LD decay helps the breeders to point the number of markers that would be needed for GWAS by dividing the genome size on the distance at which LD is decayed [63].

In self-pollinated species like barley, the LD decay is always larger than in cross-pollinated species such as maize, therefore a lower number of markers are required to cover the genome. In barley, one million SNP markers are required to cover the barley genome in case of LD decay at 5 kbp, whereas only 57,000 SNPs are required if the decay is at 100 kbp [61]. Moreover, the LD decay varied among different barley populations with less than 1 centiMorgan (cM) as a genetic distance based on the genetic linkage for the physical distance of alleles on a chromosome which is equal to the number of recombinants divided by the total number of offspring multiplied by 100) in wild and 14 cM in cultivated

winter barley and within the genome, i.e. was higher in the centromeric than telomeric regions [64]. Therefore, high mapping resolution with dense SNPs together with great genetic diversity makes barley a promising model temperate cereal crop. LD decay can be visualized by a scattering or heatmap plots of r^2 values versus genetic/physical distances between all pairs of SNPs along a genome or chromosome or specific genomic region e.g. QTL.

The historical recombination can be estimated through analyzing the LD pattern in the population which depends on multiple factors like allele frequency, recombination rate, random mating, mutation rate, genetic drift and migration, selection, population size, and structure. In the association's panel which is selected by researchers (artificial selection process), the allele frequency is not expected to fit with Hardy-Weinberg equilibrium proportions for loci (i.e., unlike bi-parental population, genotype frequencies can not be predicted by association population allele frequencies). SNPs that are not in Hardy-Weinberg equilibrium are commonly removed from GWAS analysis [65]. The advantage of association population is that recombination events which are accumulated over generations improve the map resolution with high allele numbers. In outcrossing species, the effect of mating patterns and admixture clearly explain more rapidly LD decays compared to selfing species due to the greater effective recombination in outcrossing species [31]. The effects of genetic drift include losing rare alleles that increase LD levels in small population size. Selection can also increase LD. For example, if the mutation or recombination between the neighboring alleles happened then they both will be under selection pressure. Therefore, the selection of the association population can produce locus-specific linked alleles (selected allele at a locus) which control specific phenotype that likely appeared in LD. Generally, selection and admixture increase the LD level. Finally, migration increases LD in the population and has an impact on genetic structure in an association panel. Therefore, estimating a "structure" in the population or identifying subgroups in the panel can reduce its impact. Ignoring selection, mutation, migration, or drift which could have occurred during the history, lead to having alleles in linkage equilibrium (r^2 or $D'=0$).

As discussed above, the major problem of having a false association in GWAS is the population structure. The application of mixed model methods to correct the population structure using the PCA [38] or K matrix [53] in barley is commonly used [36,40,45,66] as a powerful tool for controlling the population structure and reducing spurious signals of association whereas a combination (Q + K) of these approaches appears to be the most powerful [11,67]. These approaches have successfully split the individuals of diverse barley populations into subpopulations based upon row-type (two- and six-rowed) and/or geographical origins and/or growth habits (spring and winter subpopulations). Therefore, considering the relationship matrix for the population structure correction purpose in the mixed models is an influential method that is commonly used in barley to significantly reduce the number of false-positive associations. Note, in some cases, controlling the population structure using Q + K may lead to having over-correction and then losing significant information and output.

Recently, many studies used QTL mapping and association mapping to identify and validate QTLs associated with target traits e.g. in maize [68], faba bean [48] and brassica [69]. Two populations representing two different genetic backgrounds of bi-parental and diverse populations which were genotyped using the same set of markers. The advantage of using both populations is the easy tracking of significant markers associated with the same trait in two different genetic backgrounds. There is no study in barley that includes both analyses. Therefore, it could be very useful to consider this approach in barley to genetically improve breeding target traits.

How GWAS works

To conduct a GWAS experiment, the first step is to select the population of study with a full consideration of the size of the population (minimum 100 individuals) with preference to increase the number of individuals as much as possible to avoid Beavis effects that lead greatly overestimated of phenotypic variance when the number of individuals are small e.g. 100 [70]. Then, there are three important stages for performing a successful GWAS experiment (Fig. 2); Stage I is the phenotyping in which all genotypes should be phenotyped for a particular trait or group of traits based on the objectives of the study. Accurate phenotyping is a very critical point to detect genotype-phenotype associations. Phenotyping should be repeated over replications and/or locations and/or years. The broad-sense heritability should be calculated for raw data (note, it should be calculated after removing the outliers) including all of these factors and considering $G \times E$ interaction. High heritability is an indicator that the trait is mostly genetically controlled which is important to detect the association signals. Then, the phenotypic data can be used to estimate the mean i.e. BLUE or BLUP. Because the phenotypic data are highly unbalanced in the plants, the estimation of genotypic values is mostly calculated as fixed effects (i.e. BLUE) using mixed models [71] which have been successfully used in barley [45,52,53,66].

Stage II (Fig. 2) is the genotyping in which the same set of individuals that were phenotyped should be used for genotyping using DNA molecular markers. GBS is the most frequent method used in genotyping because it generates numerous SNP markers inexpensively that cover the crop genome (e.g. wheat, barley, etc.). The GBS-generated SNPs should be filtered based on missing data, heterozygosity, and minor allele frequency. Before running GWAS, population structure should be tested in order to select the better GWAS model. The general linear model (GLM) and mixed linear model (MLM) are statistical models often proposed for performing GWAS (Fig. 2). The GLM does not take the population structure-related into account. Hence, GLM was used in populations which did not have the population structure in faba bean, *Vicia faba* L. [62] and rice [72]. The MLM, on the other hand, considers the population structure in its model (Kinship or kinship + Q matrix + PCAs).

Finally, the phenotypic and genotypic data are combined using appropriate software (e.g. TASSEL) by which alleles associated with a particular trait can be detected after the GWAS model was selected (Stage III: Fig. 2). Phenotyping is highly recommendable to be conducted before genotyping especially for those populations with no prior information. For example, if a population consisted of 400 genotypes which were collected from different regions and the objective is to test them in a particular environment. It is possible that many genotypes could be lost due to poor adaptation to the phenotyping environment. Therefore, time and money (for genotyping) can be saved by testing the phenotypic diversity of that population first.

The significance of marker-trait associated (passing the threshold e.g. $-\log_{10}$ p-value 3) is usually determined by the false discovery rate (FDR) or Bonferroni correction (BC) which can be defined as multiple comparisons that can be fit to test the significance of hundreds of thousands to millions of markers in GWAS. For BC, the significant level is divided by the number of tests (markers) at each locus. The BC method is intensively used e.g. [73,74] to define the threshold of significant markers for several traits at once. As a result, a fixed BC P-value will be generated using the following equation

$$P - \text{value (BC)} = \frac{0.05}{K}$$

where k is the total number of markers (statistical tests).

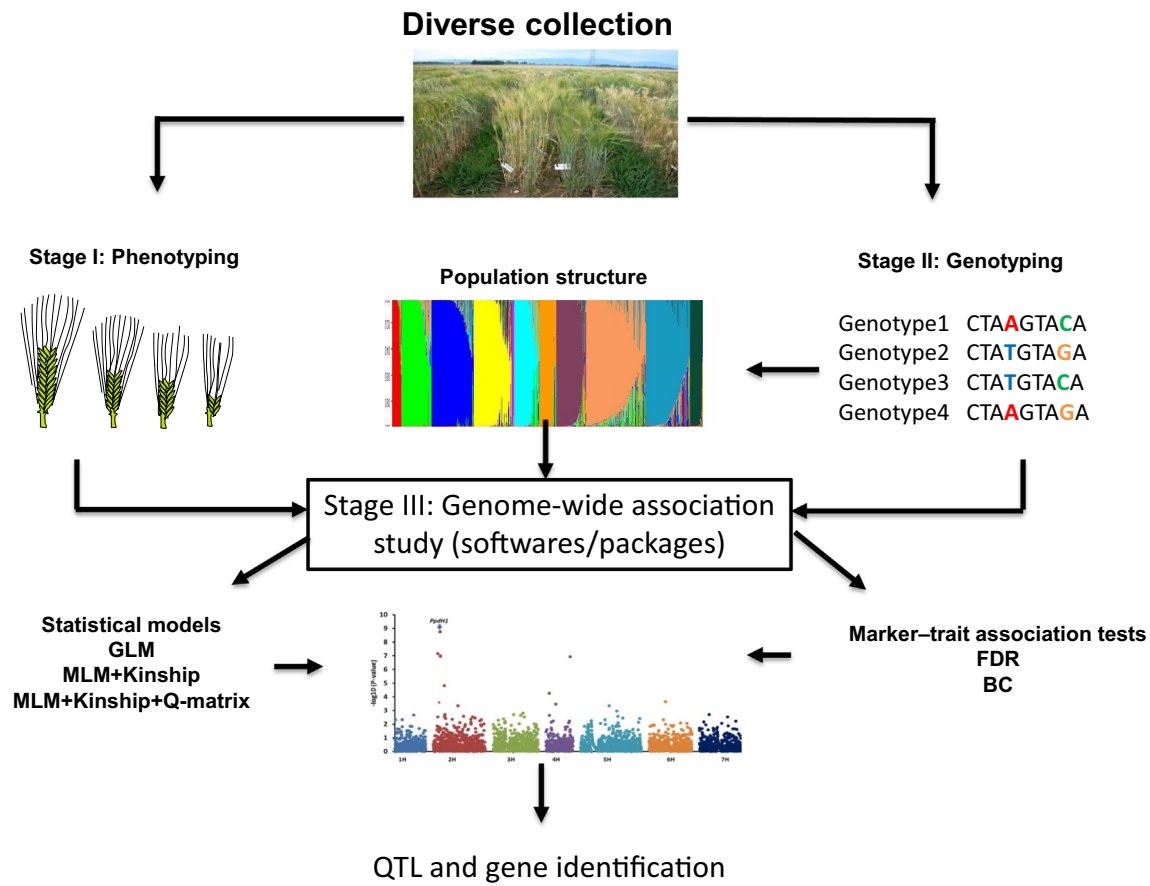


Fig. 2. The most important three stages for performing a successful GWAS experiment. Stage I: Phenotyping, stage II: Genotyping and stage III: Genome-wide association study including statistical models, multiple-testing analyses, and software/packages for QTL and gene identification.

The false discovery rate (FDR) is another test that provides an estimate of the number of actual true results among those called significant [75]. In this test, the p-values of all markers generated from GWAS are sorted in ascending order. Then, each p-value at each locus is given a rank (R - e.g. 1, 2, 3, ... 100,000). The p-value of FDR for each marker is calculated as follow

$$p \text{ value (FDR)} = \frac{pR \times 0.05}{K}$$

where pR refers to the rank of marker p-value

FDR is calculated for each trait independently, which makes it more powerful in studying the genetic factors of developmental and agronomic traits in crop plants. Compared to the fixed p-value (BC) for all traits, the p-value (FDR) is more flexible and changed based on the markers and traits. Therefore, FDR is less conservative than BC and recommended to be used in crop plant association studies to detect the highly associated markers for each trait independently. In both tests and at each locus, if the p-value of FDR or BC is less or equal to the p-value, generated from GWAS, of the marker, the association is true and the marker is associated with the trait. The marker-trait association can be tested at the significance level of 0.01 and 0.05 [38,53]. However, some association analysis studies tested the marker-trait association by using FDR at 20% of significance level as it can detect significant markers with minor effects [62]. The determination of significance level for marker-trait associations in GWAS is based on the study, which may use high FDR to investigate the whole picture of the genetic architecture of a trait or low FDR to identify candidate loci/gene (s) for further genetic and molecular studies [39].

Software for performing GWAS (TASSEL, GenStat, PLINK, and R (GAPIT))

GWAS can be performed using many software statistical packages (Stage III: Fig. 2). Here, we focused on the most important association analysis software packages that are frequently used.

TASSEL (Trait Analysis by Association, Evolution, and Linkage) is the most common software for GWAS in plants. It includes many powerful statistical methods for performing GWAS including GLM and MLM [76]. TASSEL can analyze the population structure using kinship and PCA. LD is included also in TASSEL. The software is always used in association analysis in barley e.g. [40]. The new version of TASSEL (TASSEL 5.0) can analyze genetic diversity and perform SNP calling from GBS data. Interestingly, the software includes many visualizing tools which can be used to present data such as a scatter plot of PCA, LD, Manhattan plot for GWAS results, the heat map for genetic distance, a phylogenetic tree using archaeoptery in addition to the phenotypic variance explained by markers (R^2). The new version also includes some useful data summaries, which provide a quick view for a researcher on genotypes, markers, heterozygous, missing data and number of markers on each chromosome. Old versions of TASSEL such as TASSEL v.2.1 can accept any type of DNA markers (e.g. SNP, SSR, AFLP, RAPD, etc.). The TASSEL v.5.0 accepts only SNP markers. TASSEL is free software and can be downloaded from <http://www.maizegenetics.net/tassel>.

GenStat for Windows Edition is another statistical software that can perform marker-trait association analysis in a genetically diverse population using bi-allelic and multi-allelic markers. Using GenStat, GWAS can be done either GLM or MLM models with

population structure correction to control genetic relatedness by PCA or Kinship. There is an option to define the threshold of the significance of $-\log_{10}(p)$ of which Bonferroni can be selected. Interestingly, LD decay can be determined and visualized by GenStat software and the effect of each SNP can also be calculated to show the impact of the SNP on the traits. LD decay is important to determine the number of markers required for GWAS. Plots for GWAS profile of the $-\log_{10}(P)$ of the test statistics and the map with the location of the detected significant markers, and Q-Q can also be visualized. Therefore, GenStat has been intensively used to detect causative allele(s)/loci in barley [11,36,67,77] of which had been cloned (Table 2). The GenStat software can be purchased and downloaded from <https://www.vsni.co.uk/software/genstat/>.

PLINK allows the study of a large dataset of phenotypes and genotypes [78]. It is free software that can be downloaded from <http://zzz.bwh.harvard.edu/plink/>. It provides many characteristics and features of which, PLINK performs analyses for population stratification detection, basic association tests, meta-analyses, and some other tests such as gene-based tests for association and screening for epistasis. Graphical images for Manhattan plot, Q-Q plot, and multidimensional scaling (for population structure) can be illustrated. Also, the results of GWAS and LD among SNP markers can be presented in tables produced by PLINK.

The recent advances in R statistical environment free software (<https://www.r-project.org/>) provide many useful packages for performing GWAS. The genome association and prediction integrated tool (GAPIT) is a useful R package that performs GWAS and genomic selection. The main advantages of GAPIT are: it can handle a large amount of data (SNPs and genotypes) and it reduces computational time without compromising statistical power [79]. The package includes many statistical methods such as MLM, population parameters previously determined (P3D), and efficient mixed-model association (EMMA). The results of GWAS results can be illustrated by Manhattan plots, quantile–quantile (QQ) plots and a table, including p-value, minor allele frequency, sample size, phenotypic variance explained by markers R^2 and adjusted P-value following a false discovery rate [75]. Similarly, the results of kinship are presented in a heat map and a table. Moreover, heritability estimates and likelihood function can be produced in graphs at different compression levels. Due to the aforementioned features, GAPIT becomes the most powerful and useful tool for association analysis in barley [41,45] or other cereals like wheat [80].

There is a clear trend of using GenStat for QTL and candidate-gene identification because it is one of the earliest software to do these analyses and has many features that are not available in other software. For example, by GenStat the phenotypic and genotypic data can be analyzed, BLUE values can be calculated, LD can be measured, and population structure with PCA and kinship can also be calculated and then GWAS by applying either GLM or MLM can be done. The output includes all of the important plots and information about the marker-trait associations e.g. the effect value of marker on the trait in addition to $G \times E$ interaction. Finally, the significant associations can be validated by Bonferroni correction. In other software/packages, often each step needs to be calculated separately for GWAS.

The output results of GWAS

Each software program gives slightly different parameters as output results for GWAS. TASSEL software is a good example of producing many parameters that help to dissect the genetic basis of the target trait. These parameters include the p-value of each SNP which is important to determine the significance with the trait, R^2 (phenotypic variation explained by marker) that determines if the significant SNP is a minor or major QTL, and allele effects of the significant SNP (increased or decreased the trait).

The main output can be presented in the Manhattan plot that illustrates, on a genomic scale, the P-values of all markers used in GWAS. The x-axis represents the genomic order by chromosome and position on the chromosome, while, the y-axis represents the $-\log_{10}$ of the P-value of each marker (equivalent to the number of zeros after the decimal point plus one). The associated significant SNP (lowest significant p-values), representing QTL tend to show up as a strong signal on the Manhattan plot (Fig. 3A). The threshold of $-\log_{10}$ (p-value) can be fixed at a confidence value of which $-\log_{10} \geq 3$ is the most common and reliable value (Fig. 3A). For further analysis, the threshold can be recalculated using the multiple comparison analysis that makes the p-value of SNP more robust and trustworthy (Fig. 3A).

Another important graph in GWAS is the QQ plot which illustrates the relationship between the observed and expected p-values. It depicts the deviation of the observed P-value of each SNP from the null hypothesis. The QQ plot can be used to compare the observed vs, expected values among GWAS statically models to

Table 2
Candidate-gene based GWAS which has been validated and cloned.

Population	Sample size	Growth habit	Population structure	Model	Marker info	Phenotype	Software	Candidate gene	Validation	Ref.
Genobar	224	Spring	Row-type; photoperiod responses	MLM	9 K SNP	Tillering, plant height	GENSTAT	<i>Ppd-H1</i> , <i>VRS1</i>	Mutant analysis Molecular, transcriptome, histological analyses	[38] [39,84]
						Leaf area		<i>HvCO1</i> , <i>BFL</i>		
European barley UK cultivars	138 500	Winter Spring, Winter	Row-type; seasonal growth habit	MLM	9 K SNP 1.5 K SNP	Agronomic traits	GAPIT-R GENSTAT	<i>VRS2</i> <i>Ppd-H1</i>	Map-based cloning Histological analysis	[56] [41]
						Leaf size		<i>ANT2</i>		
Western Europe and North America UK cultivars	190 401	Spring	Row-type; seasonal growth habit	MLM	2.5 K SNP 5.2 K	Spikelet fertility, spike architecture and tillering	GENSTAT	<i>INT-C</i>	Re-sequencing & Cloning	[81]
European barley	804	Spring, Winter	seasonal growth habit	MLM	9 K SNP	Heading date and agronomic traits	GENSTAT	<i>HvCEN</i>	Re-sequencing & Cloning	[11]
USDA	2,671			MLM	9 K SNP	Salt Tolerance	GAPIT-R	<i>HKT1</i> ; 5-like	Re-sequencing & Cloning	[82]

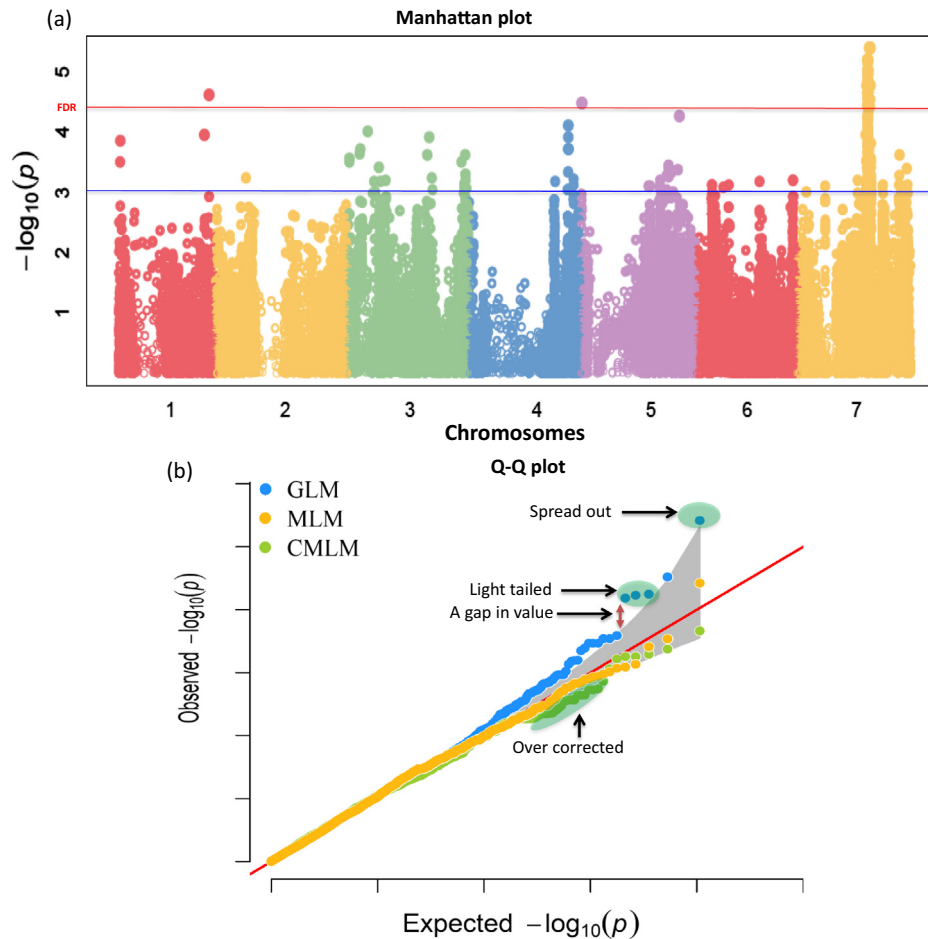


Fig. 3. The output results of GWAS. Manhattan plot (a). Horizontal-axis represents the position of markers over the barley chromosomes and vertical-axis represents $-\log_{10}(P\text{-values})$ of the marker-trait association. Each dot denotes marker. Horizontal blue-line represents threshold of $-\log_{10}(0.001)$ and red-line represents the threshold of $-\log_{10}(p\text{-value})$ passing false-discovery rate (FDR). Quantile-quantile (QQ) plot of different GWAS models (b). The plot shows the expected vs. observed $-\log_{10}(p\text{-value})$ of each marker (dote). Red-line is the standard relationship among markers. General linear models (GLM), mixed linear models (MLM) and compressed MLM (CMLM).

show how well the model used in GWAS considering the population structure and familial relatedness and then can be applied, for instance, MLM compared to GLM or CMLM models (Fig. 3B). The diagonal or standard line (red in Fig. 3B) shows whether the points are matched perfectly or deviated which reflect the distribution. Gray area shows 95% confidence region for values. It is expected that most of the data points in the QQ plot will lie on the diagonal line since they are not associated with the trait. Whereas the deviations from this line suggest that the model does not sufficiently control the population structure which can be interpreted as spurious associations.

There are three main possible QQ plots, each with its own meaning:

- (1) the observed values correspond to the expected values, all points (observed vs. expected p-values) are very near or on the diagonal line and within the confidence interval, the gray highlighted region (Fig. 3B).
- (2) the significant SNPs (observed p-values are highly and significantly different from expected p-values under the null hypothesis) move towards the y-axis (Fig. 3B).
- (3) If there is an early separation of the points or unclear trend, this means that the results could be due to unaddressed population structure or/and poorer quality of the phenotypic data. In this case, most of the highly deviated SNPs are

represented as a false association and other considerations (e.g. correction of population structure, phenotypic data correction) are required (Fig. 3B).

It is implausible that GWAS will completely explain the heritable proportion of complex traits, but, it can explain a large proportion. The difficulty in detecting small effects by rare variants or very small effects by common alleles makes it impossible.

GWAS as a driver of gene discovery in barley

In barley, progress has recently been made toward identifying loci/genes underlying the phenotypic and allelic variation of complex traits using GWAS with a high throughput SNP platform, i.e. sufficient marker density to cover the entire genome. Many studies have demonstrated the power of association population mapping in identifying candidate genes that control the target traits (Fig. 4). Here, we will also demonstrate the power of GWAS in detecting the allelic variation which had been functionally validated (Table 2).

ANTHOCYANINLESS 2 (ANT2) is the first gene in barley discovered using GWAS and then cloned by Cockram et al. [67]. Re-sequencing *ANT2* candidate basic helix-loop-helixprotein1 (*HvbHHLH1*) gene showed the deletion in a premature stop codon

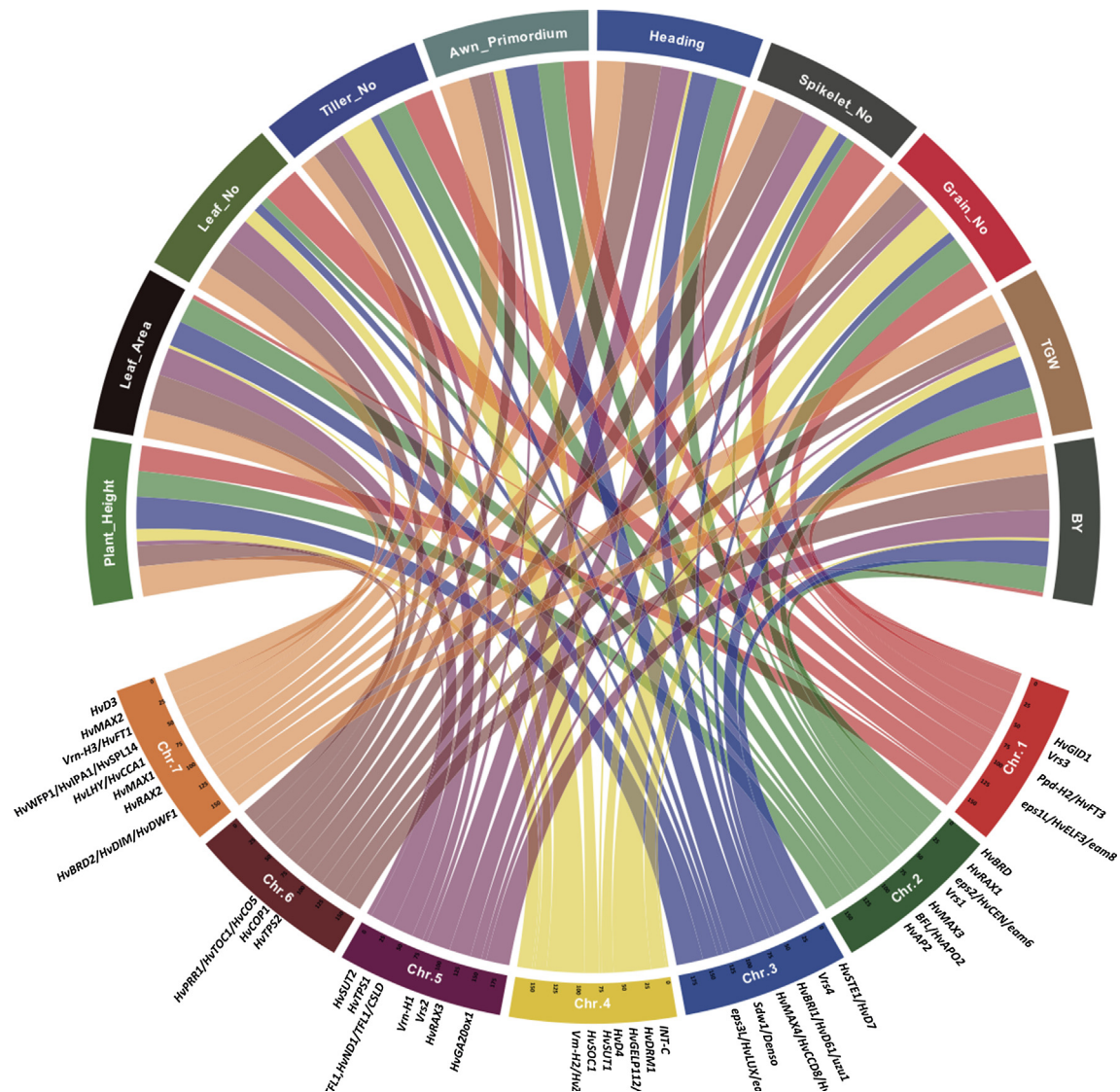


Fig. 4. Summary of the most important genes distributed over barley chromosomes, which are involved in developmental and agronomic traits.

upstream that lead to lack of anthocyanin in the tested mapping population. Lateral spikelet fertility gene *INTERMEDIUM-C* (*INT-C*) is another example. It is an ortholog of the maize domestication gene *TEOSINTE BRANCHED 1* (*TB1*) and was detected by GWAS [81]. The natural allelic variation at this locus shows the positions of the 17 independent *int-c* mutant alleles, which is important to understand the genetic basis of crop domestication and fundamental spikelet developmental processes. *HvAPETALA2* (*HvAP2/Cly1*) had been also detected by GWAS that is associated with the genetic basis of natural variation of spike density-related traits in spring barley [77]. In the same study, the GWAS was used to discover the *ZEOCRITON* alleles which are associated with the studied traits in a 401 two-rowed UK spring barley population. In 2012 Comadran et al. [11] developed the 9 K iSelect Illumina™ SNP platform from which they provided a high GWAS map resolution that leads to cloning barley *CENTRORADIALIS* (*HvCEN/eps2*) which contributed to the spring growth habit and environmental adaptation in cultivated barley. Using a GWAS, the genomic region of salt tolerance gene *HKT1;5* had been discovered in barley and then re-sequencing the gene that is responsible for Na⁺ unloading to the xylem and controlling Na⁺ distribution in the shoots validates it [82]. The aforementioned successful examples of genes identified

through GWAS provides strong evidence that GWAS served as part of a rapid gene-cloning strategy that can be effectively used for further gene cloning.

QTL and allelic variation detected using a GWAS

GWAS is also used to explore the important alleles of the candidate genes underlying the natural variation (Table 2 and Fig. 4). For example, GWAS analysis in a worldwide barley collection discovered the most important alleles of *Six-rowed spike 2* (*VRS2*) gene. Haplotype 4 encodes a functional *VRS2* protein showed a significant and consistent association signal for phase duration, leaf area, and leaf and tiller number [56]. The natural variation of the pre-anthesis stages/phases in barley is based on the genetic allelic diversity at *PSEUDO-RESPONSE REGULATOR* (*HvPRR37*)/*PHOTOPERIOD RESPONSE LOCUS1* (*Ppd-H1*) gene, as the central heading time gene, that in turn regulates the responses to long-day photoperiod. A single nucleotide change at marker 22 alleles (G/T in the CCT-domain) *Ppd-H1* gene changes the status of the photoperiod responsive (*Ppd-H1*) accessions to reduced photoperiod sensitivity (*ppd-H1*) to long-day conditions. The change led to the evolution of

the late heading of European accessions (mostly cultivars/breeding lines) compared with the early heading of landraces (carrying sensitive alleles *Ppd-H1*) and coming from the barley center of origin [36,83]. Histological analysis confirmed the GWAS results that allelic variation at marker 22 of *Ppd-H1* controlled cell proliferation period and leaf maturation which directly contribute to leaf size in European winter barley [41]. In other studies, GWAS demonstrated that the genotypic variation at the barley domestication gene *VRS1* influenced the leaf area and tiller number which was confirmed by mutant, histological, transcriptome and molecular validation in case of leaf area [84]. GWAS showed that natural selection of adaptive evolution for late heading in European accessions improves other adaptation and developmental traits such as increased leaf area and tiller number which in turn improve grain yield [38,39,41]. Many other genes found to be associated with important developmental and agronomic traits (Fig. 4) of which *BFL* (*BARLEY FLORICAULA/LEAFY*) gene had strong associations with phase transitions, tillering and other yield-related traits [36,38].

GWAS demonstrated its strength to detect novel loci/QTL of natural variation (Table 3). To this end, many barley populations and marker types have been used for studying the genetic basis of wide-range of traits including agronomic traits (Table 3). For example, Genobar world wide spring barley collection consists of 224 accessions had been intensively used for GWAS studies that revealed QTL (Table 3) for tillering at 5H (31.7–34.1 cM) and 6H (16.9–24.6), plant height at 5H (21.3–24.6) [38], leaf area at 1H (95.9–97.9) and 2H (50.9–56.4) [39] in addition to many other novel QTL underlying the natural variation of germination, seedling architecture at 1H (76–48), 2H (112–115) and 5H (44–45) [52], phase transition and developmental stages 3H (56–64) and 5H (2.6–9.3) [36]. Natural variation analyses in a NAM population (consists of 1420 individuals) were applied that provide further insights into the evolutionary genetics underlying adaptive traits in barley [57,66,73,74]. Using NAM population, the allelic variation at many genes involved in phase transition and development (*Vrn-H*, *Ppd-H*, and *Denso*, Fig. 4) in addition to many other novel loci (Table 3) at 4H (3–4 and 110–114) have been detected [57]. Whereas Saade et al. [73] discovered the locus underlying biodiversity in leaf sheath hairiness at 4H, 111.3 cM using the same population (Table 3). GWAS approach was used to understand the genetic mechanisms of biotic and abiotic stress in barley. For instance, salt and drought stress tolerance alleles/loci were discovered in different diverse barley populations [52,66,85]. The genetic basis of seedling root and shoot architecture under drought stress had been studied that reveal new loci at 1H (76–48), 2H (112–115) and 5H (44–45) with the putative candidate genes [52]. In other studies, GWAS had been conducted to explore the genetic factor (Table 3) underlying drought tolerance agronomic traits e.g. grain yield, TGW, peduncle, leaf, and spike length [86] and physiological parameters e.g. water use efficiency and water content among 2H (118–119), 3H (24–25), 4H (49–55) and 5H (48–49 and 147–148) are important [87]. Studies focused on discovering the natural variation of salt tolerance in barley and effective loci at 2H, 140–145 [66] and 2H (3.5), 4H (1.45) and 5H (43.5) have been detected [88]. Novel loci associated with natural variation of resistance to stripe rust, Fusarium, the net form of net blotch and stem rust [89–93] whereas the study by Turuspekov et al. [94] identified highly significant QTL at 6H (63–64) for stem rust resistance (Table 3). Nagel et al. [95] used GWAS for the first time to study seed dormancy and pre-harvest sprouting traits and revealed novel loci at 1H (5), 3H (104.3 and 135.6) and 5H (169.4). In addition to above-mentioned studies, many others have been conducted to uncover the genetic architecture of yield component and quality traits under field conditions e.g. at 2H (41–52 cM), 3H (8–9), 5H (100–108) [40], 2H (106–107), 7H (1.6–15) [96], 2H (145–155), 3H (95–100) and 5H (160–170) [97]. The GWAS studies in barley

detect novel QTL which not previously reported including candidate genes for further genetic and/or molecular characterization and validation. GWAS output provides new sources of alleles that enhance the diversity of tolerance to biotic and abiotic stress in addition to improving yield in future breeding.

How to predict the gene in barley?

Once the GWAS output has passed all of the statistical criteria, the next step will be candidate genes identification. The most important, consistent and significant association(s)/QTL(s) including highly significantly associated markers ($-\log_{10}$ SNPs passing the multiple comparison analysis e.g. FDR) e.g. genomic region at 7H (Fig. 3A) will be selected to find their physical positions using recently published barley genome sequence [98]. The physical map can be used to define the physical interval on the genome (using flanking SNPs of association/QTL within the LD decay physical distance) that can include candidate gene(s). **BARLEX**, IPK server <http://apex.ipk-gatersleben.de/apex/f?p=284:10> offers such information about the SNPs and barley genes including their annotation GO Terms and other useful information.

In case there are many highly associated SNPs within the LD decay physical distance, we recommend narrowing down the physical interval up to hundred(s) Kbp that empowered us to detect the closest candidate genes to the highly associated SNP. In the best case, SNP(s) can be physically within the candidate gene that leads to checking whether these SNP(s) are functional i.e. having a significant impact on the associated traits or not.

Here we suggest selecting the high confidence gene including SNPs within their physical position to make “**SNP-Gene based haplotype analysis**” that allows us to validate the functionality of the SNP(s) within the candidate gene. This analysis can identify which SNP has the most effect on the associated trait(s) by splitting the population based on the alleles of each SNP. Matching the allele with the phenotypic value in the population and then statistical analysis for the significant differences test between alleles e.g. *t*-test statistics can show the importance of each allele on the targeted trait(s).

Together with the Exome Capture Sequence, data and gene expression available at **BARLEX** for barley will allow one to check whether the candidate gene is a capture target and then reveal the most promising haplotypes underlying associated traits, in addition, to check in which organ and at which developmental stage the gene is expressed. This analysis will provide the ability to build phylogenetic-trees and haplotype-networks within and between individuals and subpopulation or genetic background (e.g. geographic region) to provide insights into the evolution of the candidate gene(s), potentially providing evidence for selection in particular regions/environments. This approach helps further functional and molecular analysis e.g. mutagenesis, expression analysis and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) (CRISPR)/Cas9 (CRISPR-associated protein9). The analysis will validate and provide trait-enhancing alleles for crop breeding/genetics and shed new light on evolution and functions.

Genomics of polyploidy cereals

Ploidy level in crop species is a significant factor influencing the genotyping qualities, SNP discovery, and validation. Many cereal crop species are unlike barley and rye with a diploid genome ($2n = 2x = 14$). The polyploidy genome in cereal crops such as tetraploid ($2n = 4x = 28$) and hexaploid ($2n = 6x = 42$) wheat, tetraploid and hexaploid oats (*Avena sativa*), and triticale (*Triticosecale*) that can be tetraploid to octoploid ($2n = 8x = 56$). The genome complexity in the allopolyploid species as results of

Table 3
The most significant associated genomic regions with quantitative traits in barley using a GWAS approach.

Reference panel	Sample size	Marker info	Phenotype	Chr. (pos. (cM))	Software	Ref.
Genobar	224	1.5 K SNP	Heading date, plant height, TGW, starch content, crude protein	2H (41–52), 3H (8–9), 5H (100–108), 6H (28, 60, 125), 7H (1 0 4)	TASSEL	[40]
		9 K SNP	Phase transition, developmental stages, tillering, plant height, leaf area	1H (3–8, 95.9–97.9), 2H (50.9–56.4, 82–88, 141–147), 3H (56–64, 122–127), 5H (2.6–9.3, 21.3–24.6, 31.7–34.1, 83–86), 6H (16.9–24.6)	GENSTAT	[36,38,39]
			Germination and seedling shoot and root architecture traits	1H (76–48), 2H (112–115), 5H (44–45)		[52]
European cultivars	183	253 DArT & 22 SSR	TGW, glume fineness, extract and friability	1H (116–123), 5H (120–127, 132–134)	TASSEL	[110]
German winter	106	1,169 DArT	Grain yield, TGW, agronomic and quality traits	2H (47–48), 3H (51–53), 6H (46–47, 142–143), 7H (1–5)	TASSEL	[111]
Barley Germplasm	185	710 DArT, 61 SNP and 45 SSR	Drought tolerance related traits (Grain yield, TGW, peduncle, leaf, and spike)	3H (1 5 3), 5H (139–150)	GENSTAT	[86]
European cultivars	174	839 DArT	Grain yield, TGW, agronomic and quality traits	1H (10–12, 94–96), 2H (133–136), 5H (13–15)	GENSTAT	[112]
Worldwide	206	408 DArT	Salinity tolerance	2H (3.5), 4H (1 4 5), 5H (43.5)	TASSEL	[88]
UK cultivars	500	1.5 SNP	Auricle, awn, spike, rachis, spikelet and grain-related traits	2H (82–90), 4H (106–119), 5H (100–113, 119–130)	GENSTAT	[67]
Pan-European Barley Cultivar Collection	379	9 K SNP	Grain yield-associated traits	2H (110–115, 145–155), 3H (95–100), 5H (160–170)	GENSTAT	[97]
Jordanian landraces	150	9 K SNP	Harvest index & Spikelet number per spike	2H (106–107), 7H (1.6–15)	TASSEL	[96]
NAM	1,420	9 K SNP	Leaf sheath hairiness.	4H (111.3)	SAS	[73]
			Salinity tolerance	2H (140–145)		[66]
			Heading time and yield related-traits	4H (3–4, 110–114)		[57,74]
Kazakhstan collection	92	9 K SNP	Stem rust resistance	3H (131–136), 6H (63–64)	TASSEL	[94]
EcoSeed	184	9 K SNP	Seed dormancy and pre-harvest sprouting	1H (5), 3H (104.3, 135.6), 5H (169.4)	GENSTAT	[95]
Modern European cultivars	148	407 SSR	Spike length, plant height and grain number	1H (64–65), 2H (3–4, 14–15), 3H (126–127), 5H (86–87, 130–131), 6H (44–45, 95–96)	TASSEL	[85]
Drought tolerance collection	109	5,153 DArT	Water use efficiency, water content and relative water content	2H (118–119), 3H (24–25), 4H (49–55), 5H (48–49, 147–148)	TASSEL	[87]

hybridization of related species e.g. triticale (a cross between wheat (*Triticum turgidum*) and rye (*Secale cereale*)) [99] makes genotyping far behind the diploid crop species and slowed down SNP discovery. A notable challenge in polyploid cereals is how to assemble and distinguish the homologous SNPs among the genotypes and subgenomes and/or paralogous SNPs due to duplicated copies and transposable element. High sequence similarity among the subgenomes impedes discovering the homologous variations which are important for understanding the genetic factor underlying the quantitative traits in polyploid crops. The redundancy of homoeologs among the subgenomes can curb important phenotypic variation. Genetic studies in polyploid cereal crops required high-density, quality and number of SNPs through high-throughput SNP genotyping to overcome on complexity and size of genomes. For instance, SNP arrays achieved noticeable progress in SNP polymorphic rates and size had been developed in wheat 9 K, 90 K, 35 K, 135 K and 820 K SNP [100]. Prior to attempting GWAS in polyploid crops, some important points must be carefully considered, e.g. how to detect and define the rare alleles, to which subgenome the allele belongs and how to deal with the effect of population structure and which subgenome contributes more to the structure and variation in addition to LD and its decay. The recent advances in the genome sequence in allopolyploid crops e.g. bread wheat and its diploid and tetraploid progenitors [101–104] help the researchers in distinguishing among homologous copies carried by subgenomes. The new technologies lead to *de novo* assembly of the chromosomes that reduce the complexity by assembling the highly redundant genome and assign the genes. Sequencing ancestral diploids aid the assigning of specific sequences to the diploid progenitor. The emergence of the pan-genome in crops will improve the sequence coverage and quality. It will lead to develop a core genome that contains all shared sequences in all sequenced individuals and add the absent/present genes [105].

In wheat, the **Wheat@URGI** (Unité de Recherche Génomique Info) databases and tools to discover genetic and genomic wheat data according to **IWGSC** reference sequences, physical maps, genetic maps, polymorphisms, genetic resources, phenotypes and arrays [106]. It maintained and hosted by a research unit in genomics and bioinformatics at Institut National de la Recherche Agonomique (INRA). WheatMine website <https://urgi.versailles.inra.fr/WheatMine/begin.do>.

It is also possible to blast the targeted sequences at the specific chromosome and ancestral diploid using blasting server https://urgi.versailles.inra.fr/blast_iwgsc/blast.php.

The output contains the gene description including annotation, GO Terms and other useful information. High-quality reference genomes and gene discovery, using high quality *de novo* genome assembly improve the GWAS analysis to discover the candidate gene using the physical position of linked SNP markers with the natural variation. Using SNP arrays for genotyping, researchers working on wheat were able to detect candidate genes for grain yield-related traits [46,47,80,107,108].

Even though promising progress in polyploidy cereals genomics analysis has been made in last few years, most genetic studies in polyploids e.g. wheat have so far relied on diploid models i.e. barley to simplify the polyploid data and overcome of the aforementioned obstacles in wheat. Barley has been a model for genetic and cytogenetic studies in last decades due to many features e.g. low chromosome number, dozens morphological and cytological mutants, thousands of genetic stocks, genetic mutant stocks for reverse genetics approaches e.g. Targeting Induced Local Lesions IN Genomes (TILLING) are available. In addition to advances in gene editing e.g. Cas9 and its close evolutionary distance and extensive conservation of synteny, barley is a useful genomic model for wheat and other polyploidy cereals.

Future applications of GWAS strategy in crop improvement

The knowledge of natural variation in barley as a model crop for small grain cereals has advanced tremendously in the past years. In the near future, GWAS in barley will be more informative using the advances in the genomic sequence, high-throughput SNP genotyping with the impressive set of genetic resources that are available at the genebank e.g. IPK. The GWAS output can be implemented and used in many aspects, for instance, breeding, genetic mapping, candidate genes, and gene editing. Highly accurate phenotyping by researchers or high-throughput phenotyping platforms will also increase the power of GWAS in detecting novel loci. Such advances provide resources that improve and facilitate breeding, genomic and genetic analysis of important agronomic traits in crops. More deep analysis for the detected causative loci by GWAS e.g. haplotype-based analysis is a key for genomics-assisted crop breeding. GWAS has a higher resolution because of more recombination events, and more genotypes can be used for a broader genetic base compared with biparental QTL mapping. GWAS in future barley work should be considered as an exploratory analysis for the right selection of true segregating parents that can be used in the QTL mapping population and for further genetic and molecular validation of the associations. GWAS can also used to understand breeding-program variation (the genetic variation in the association panel used to develop improved plant material) or marker-assisted selection (selection of individuals for breeding program based on their genotypic information at specific allele linked to QTL) because the association mapping population can be considered as a source of alleles which are not or rarely present in the bi-parental mapping populations.

Recently, many studies used QTL mapping and association mapping to identify and validate QTL associated with target traits e.g. in maize [68], faba bean [48] and brassica [69]. This approach using both populations to check whether the significant markers associated with the same trait in two different genetic backgrounds or not [109]. There is no study in barley using this approach; therefore, it could be very useful to consider it in barley to genetically improve breeding target traits. The association mapping population is a source of allelic variation including the domestication alleles which are mostly present in wild relatives and landraces offers an excellent resource to increase the discovery of functional loci/genes underlying genetic variation for complex traits including yield, disease resistance, and abiotic stress tolerance. The analyses allow predicting the function of many alleles representing mutations and candidate genes which have an agronomic impact, hence can be used in further molecular validation e.g. gene expression and gene editing. With the help of statisticians and bioinformaticians, the analysis of complex traits in crops will be much improved through developing more databases and statistical models. Integrating -omics and genetics will be crucial for crop improvement and molecular analyses. The extension of the analysis of natural variation to the molecular mechanism will elucidate the mechanisms involved in barley plant development and adaptation.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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