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Mapping and exploiting the barley genome – techniques for mapping genes and relating them to desirable traits

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Abstract:

Barley is the fourth most cultivated cereal in the world and is thus an important crop in breeding. A large variety of traits are needed for resistance to various stresses as well as to meet the requirements of its several uses. To breed efficiently for those traits, identification of the causal genes and availability of genetic markers for selection are important targets for research. Gene mapping approaches have undergone radical changes in the past decades. In this chapter, we give an overview of these changes emphasizing the importance of the availability of barley reference genomes and sequencing technologies. An overview of the methods and resources for mapping barley genes is given, covering developments over time, and concluding with future trends.

Key words:

Gene mapping, markers, sequencing, genome

Content:

1 Introduction

Barley has been proposed as a model species for Triticeae (Linde-Laursen et al., 1997) given its high economic importance, being the fourth most cultivated cereal (FAOSTAT, 2018) and at the same time having a less complex genome than the tetra- and hexaploid wheats. It is diploid and highly inbreeding, and a large number of barley genetic stocks are available. Barley can adapt to various biotic and abiotic stresses and has various uses, from malting to animal feeding, thus breeders target

a large number of traits. The molecular mapping of genes and traits started more than three decades ago and, since then, genetic and genomic resources for barley were systematically developed and improved. Marker-assisted selection was implemented to accelerate breeding, however, low marker density and only loose linkage of markers to functional genes were serious limitations in early attempts to isolate functional genes underlying traits of interest. The complexity of the 5.1 Gbp barley genome, packed full with repetitive elements (International Barley Genome Sequencing Consortium, 2012; Mascher et al., 2017) rendered this step slower than in smaller genome plant species like *Arabidopsis* or rice. The first gene in barley isolated by positional cloning was *Mlo*, a gene conferring resistance to powdery mildew (Büschges et al., 1997). As of today at least 65 cloned barley genes have been documented, including 28 for architecture traits alone (Hansson et al., 2018). Forty-two of them were cloned after 2010, demonstrating the importance of improved genomic resources in barley gene isolation. The introduction of next-generation sequencing (NGS) technologies constituted a paradigm shift, bringing barley genetics into the genomic era where marker discovery and genotyping is easy and fast. Recently, an annotated reference sequence was published (Mascher et al., 2017), enabling a range of new methods to rapidly and precisely map genes at moderate cost, and to provide breeders with tools for genomics-assisted breeding. Species diversity and germplasm collections are key for finding new diversity for traits desirable in breeding but, despite the careful survey of the species that started centuries ago, genebanks' descriptions remain limited. NGS also opened the doors to their complete genetic characterization, providing better-informed access and use of genetic resources in breeding programs.

In this chapter, we review the methods currently available for mapping genes and quantitative trait loci (QTLs) in barley, from high-throughput genotyping to gene identification, with a focus on how the application of NGS technologies in barley studies can accelerate gene mapping. We conclude by considering prospects for the future.

2 What are your markers? New possibilities in the genomics era

When an interesting phenotype has been identified, the first steps are to determine whether it is mono- or polygenic, and its dominance. This is achieved by studying the segregation of the trait by phenotyping a segregating population, typically derived from crossing homozygous parental genotypes. Segregating progeny are then genotyped to map the gene. Genetic markers used in gene mapping have undergone constant progress for almost fifty years. From the use of metabolites, allozymes and isozymes, DNA markers emerged almost 40 years ago, allowing higher marker density and flexibility.

The first DNA markers were restriction fragment length polymorphisms (RFLPs) based on fragment length differentiation of DNA digested by restriction endonuclease (Botstein et al., 1980; Graner et al., 1991; Tanksley et al., 1989). For visualization, a Southern blot of the digested fragments was hybridized to a radioactively labeled DNA probe and then exposed to x-ray films, resulting in differential banding profiles of the autoradiogram. Widely used in the 1980s, hybridization-based markers entailed laborious procedures and labeling of probes with radioisotopes. The development of PCR technology in 1985 (Saiki et al., 1985) prompted the replacement of those markers by PCR-based markers (Figure 1). Initially, RFLP DNA fragments were sequenced (Michalek et al., 1999) and converted into PCR-based markers like sequence tagged site markers (STS) (Olson et al., 1989). Since this time, the development of alternative PCR-based assays like random amplified polymorphic DNA

(RAPD) (Kleinhofs et al., 1993; Williams et al., 1990) or amplified fragment length polymorphisms (AFLP) (Becker et al., 1995; Vos et al., 1995), both of which are more frequent in the genome, diminished the importance of RFLPs and their derived STS-markers for genetic analysis. However, those new markers were also labor-intensive and poorly reproducible in the case of RAPD (table 1). The development of microsatellites (or simple sequence repeats, SSR) (Becker and Heun, 1995; Litt and Luty, 1989; Saghai Maroof et al., 1994) was another PCR-facilitated breakthrough for genotyping. Based on the length of a tandemly repeated nucleotide motif of 1 to 6 bases, SSR markers have been the markers of choice for trait mapping for years and are still used in numerous current projects because of their advantages: abundant, codominant, polyallelic universal, robust, easy to automate and transferable between genotypes (Gupta and Varshney, 2000).

Figure 1: Cumulative number of papers per year per type of marker. Based on the number of papers in PubMed using the word “barley” as well as the name of the different markers in their title or abstract.

Genotyping entered the high-throughput era with diversity array technology (DArT) (Jaccoud et al., 2001), developed in 2004 for barley (Wenzl et al., 2004). This hybridization-based method generates whole-genome fingerprints by scoring the presence/absence of DNA fragments and provided genotype info at several thousand loci in parallel. DArT markers are reliable and were extensively used in several species for establishing high-density consensus maps (Wenzl et al., 2006). But, with the development of automated, multiplex sequencing technologies, and the inherent reduction of costs, higher-throughput genotyping technologies emerged. Through Sanger sequencing technology, expansive expressed sequence tag (EST) (Zhang et al., 2004) datasets were produced from different genotypes. They were gathered in databases like HarvEST (http://www.harvest-web.org/) and provided the first generic genome-wide datasets for single nucleotide polymorphism (SNP) mining and marker development, eventually leading to the development of the first SNP arrays in barley. SNPs represent the most abundant source of variation in the genome, making them very good polymorphisms for gene mapping. SNP arrays allow the genotyping of a large number of polymorphisms by array capture of total DNA, but require prior SNP discovery through DNA or RNA sequencing. Successive EST assemblies provided such a basis for the development of Illumina platforms BOPA1 and BOPA2, later merged into the Illumina GoldenGate assay containing almost 3,000 SNPs (Close et al., 2009; Muñoz-Amatriaín et al., 2011). The barley SNP array resources became precursors to higher density formats like the Illumina 9K iSelect chip (Comadran et al., 2012) and, more recently, the 50k iSelect SNP array (Bayer et al., 2017) that allows for parallel genotyping of almost 45,000 SNPs.

The SNP arrays allow for high-throughput genotyping, but their production requires a lot of resources and includes a bias toward alleles that were present in the population used for polymorphism detection. Further improvement of NGS technologies introduced an ongoing decline in costs. This provided the basis for establishing the first barley genome sequences (International Barley Genome Sequencing Consortium, 2012; Mascher et al., 2017), which enable sequence-based genotyping at the genome scale. This has removed the bottleneck of the marker discovery process and allows for direct SNP identification in the population of interest. Deep sequencing of the whole barley genome entails a heavy sequencing load, and its resolution would be higher than that needed for the purpose of SNP calling and genetic mapping. Here, several complexity reduction methods helped to introduce more cost-efficient solutions. One is to sequence only the coding fraction of the genome, either

through RNAseq or through exome capture sequencing (Mascher et al., 2013). Exome capture is based on the use of oligonucleotide baits specific to a set of exons predicted from the sequence of cv. Morex (International Barley Genome Sequencing Consortium, 2012). Those baits are then hybridized to genomic DNA to capture the respective gene space for sequencing. The region targeted for sequencing is thus greatly reduced, and sequencing load commensurately so, allowing for higher coverage sequencing and, ultimately, highly accurate genotype calling. However, even if this is an efficient method for some applications (Mascher et al., 2013), it is labor-intensive. Another approach involves randomly selecting regions for sequencing based on the presence of restriction sites, greatly lowering the cost and labor intensity. Three such methods are used in barley: RAD-seq (Chutimanitsakun et al., 2011), genotyping by sequencing (GBS) (Poland et al., 2012) and DArTseq (DArT sequencing: www.diversityarrays.com). RAD-seq consists in cutting the DNA with restriction enzymes like *Sbf*I or *Eco*RI (Baird et al., 2008). The fragments are then barcoded through ligation to adapters, and the samples from several individuals are pooled. Subsequent steps are performed on the pooled samples, decreasing the labor and cost. The pooled fragments are sheared and size-selected to achieve a length suitable to the sequencing platform, followed by a selective PCR used to select only the fragment harboring the restriction site. GBS follows a similar protocol, but with fewer steps. It utilizes two enzymes: a rare-cutter (*PstI*) and a frequent-cutter (*MspI*). Fragments are barcoded as follows: forward adaptors are designed with the *PstI* adaptor whereas reverse adaptors are matching *MspI*. Unlike RAD-seq, no shearing or size selection step is required after pooling the fragments. The short *PstI*-*MspI* fragments are instead selected by a selective PCR amplification step with a short extension time, and sequenced on an NGS platform. DArTseq has a similar protocol to GBS, but uses different enzymes in pairs with *PstI* (Wenzl et al., 2004). Both GBS and DaRTseq complexity reduction are slightly deviated to genic regions because of the use of enzymes more efficient in under-methylated regions.

The prevalence of these different methods over time is illustrated on figure 1, and their distinctive characteristics are listed in table 1. The polymorphisms detected by these sequencing-based methods can be used directly for mapping, or to design markers to genotype a larger number of plants with a lower marker density. Those markers are classically used in the process of high-resolution mapping (see part 6). The most popular ones are *cleaved amplified polymorphic sequences* (CAPS) (Konieczny and Ausubel, 1993), but newer methods like detection of SNP-specific hybridization by fluorophores is growing. Of those systems, the two most frequent are Taqman (Holland et al., 1991) and KASPar (LGC genomics, Semagn et al., 2014) markers. The genotype at a locus is determined in a PCR with pairs of fluorescently labeled primers or probes, with one of each pair being specific to an allele. After a single PCR, the reading of the fluorescence is performed in a qPCR thermal cycler or a fluorescent plate reader. The relative fluorescence levels from each member of a marker pair allows clear distinction of heterozygotes and homozygotes. These systems thus allow a rapid, reliable and efficient genotyping of a large number of samples at a limited number of loci.

Table 1: Characteristics of the most used markers in barley, currently and in the past.

3 Classical mapping strategies - still efficient but improving in the genomics era

Access to high-throughput genotyping conferred considerable benefits for linkage mapping. It accelerated data generation for mapping by generating high numbers of markers in a single

experiment, and allowed an increase in population size owing to reduced labor intensity in comparison to previous genotyping methods. Linkage mapping of a given trait is usually performed with the help of designed bi-parental mapping populations. The first step of mapping is to assess whether the trait is controlled primarily by a single major gene, or if it is a quantitative trait, likely influenced by many genes. This is determined by studying the segregation and distribution of the phenotype in developed populations. If the trait is controlled a single gene, the segregation pattern is also the way to determine the status of dominance of the gene. A trait governed by a single gene can be mapped in a simple population like F_2 or back-cross (BC). But in case of a quantitative trait, the unmonitored segregation in the numerous heterozygous regions limits the replication of phenotyping and can impact its reproducibility, ultimately reducing the power of QTL detections. Recombinant inbred lines (RILs) (Burr et al., 1988), obtained by repeatedly selfing F2 lines, were created to increase the total number of recombination events while obtaining mostly homozygous lines. Due to this last characteristic, phenotyping can then be repeated several times with a high level of reproducibility. However, the generation of this kind of population is tedious as it requires 6 to 8 generations and has encouraged the development of faster ways to obtain homozygous lines. One of these is the doubled haploid (DH) line (Clapham et al., 1973; Kao et al., 1991), which are produced by inducing haploid lines from F1 plants and converting them into diploids. DH lines are based on a single gamete, therefore they only have one meiosis to accumulate recombinations. The total number of recombination is therefore lower than in RILs, but it is the fastest way to obtain homozygous lines and is now widely used (see chapter 7).

Linkage mapping is used for both single genes and QTLs and can be performed with different algorithms. The single-marker analysis identifies the gene interval or QTLs though calculation of the effect of each marker of the trait by *t*-test or ANOVA (Beckmann and Soller, 1988; Edwards et al., 1987; Thoday, 1961), but it lacks precision. Interval mapping (Haley et al., 1992; Lander and Botstein, 1989) is based on the simultaneous analysis of two markers at a time, where the two marker positions define an interval within which the likelihood of a gene or QTL being present is estimated. The resulting position and effect of each detected locus is thus more precise. The composite interval mapping (CIM) (Zeng, 1993) approach takes into account that a trait can be influenced by several QTLs, thus reducing the error. To improve the power of linkage mapping, several statistical models, such as multiple interval mapping (Kao et al., 1999), were derived from CIM, including more factors like additive, dominance or epistasis effects.

The availability of high-throughput genotyping and access to a reference genome sequence of barley now allows better linkage mapping by increasing the number of markers included in such studies, while making reference-guided mapping possible, using the information of the reference sequence to define the order of markers. But, even if increasing the number of markers can improve the mapping resolution, this improvement is limited by the number of recombination events that occurred at the locus, and the genetic resolution depends mostly on the size of the population and the frequency of recombination.

4 The association mapping boom

The genetic resolution achieved by linkage mapping is limited. It is directly linked to the recombination frequency in the population, which remains low because of the low number of generations that can be achieved. To alleviate this problem a natural population can be used to

perform genome wide association studies (GWAS). First used in humans where experimental populations cannot be developed (Spielman et al., 1993), it also has advantages in plants traits mapping: The recombination events that can be identified in natural populations are the historical recombinations that occurred during the evolutionary history of the species, representing a much higher number of generations than the few collected in experimental populations (Zhu et al., 2008).High-throughput genotyping allows to easily retrieve a large number of polymorphisms in a population and the genomic era became the one of GWAS. This mapping strategy is detailed in chapter 8 and will only be generally described here.

GWAS can be applied for major genes but the kind of population used make it better suited to quantitative traits. It is taking advantage of the linkage disequilibrium (LD) that exists between the causative locus and genotyped loci nearby to identify small haplotype blocks associated with the phenotype of interest in the population. The resolution depends on the extent of LD in the genomic region of interest, and on the density of the genotyping. The optimal marker density is then linked to LD in the population: the higher the LD extent, the lower the optimal number of markers. One big advantage of GWAS is the use of a population that does not need to be created by crossing, saving time. However, the use of natural populations can also be a drawback as LD can be affected by the genetic structure of the population. In each subpopulation, allele frequencies evolve independently, creating spurious haplotype associations caused by the happenstance over-representation of the phenotype in a subpopulation that also bears similar genotypes at many loci---and not to any true association between the marker and the phenotype. This bias can be controlled by adding parameters like genetic structure or relatedness as cofactors to the model (mixed linear model, MLM) (Yu et al., 2006). These corrections may also increase the risk of false negative associations, as traits really linked to the population structure will not be detected. Other models like CMLM (Zhang et al., 2010), ECMLM (Li et al., 2014) or MLMM (Segura et al., 2012) were developed to try to further compensate for population structure.

In linkage mapping, the size of the population is crucial, as it counterbalances population structure. Compared with linkage mapping, GWAS usually allows a better resolution than linkage mapping but, has a reduced capacity to detect effects linked to rare variants---which for certain traits can represent the largest source of phenotypic variation. If the allele frequency at the locus is too low in the population, either the quality filter applied before the analysis may delete the informative markers (the minimum allele frequency is often set at 0.05), or the statistical power of the analysis itself will not allow detection. GWAS studies are thus better suited for either large-effect-low-frequency or low-effect-large-frequency loci.

5 Multiparental populations: the perfect balance?

To combine the advantages of biparental and natural diversity populations, multiparental populations were developed. The allele frequency and the QTL effects are higher than in a natural diversity panels while the effects of population structure are alleviated, so it is easier to detect rare variants or QTLs with smaller effect compared with classical GWAS. Compared to classical QTL mapping, having more than two parents also adds some historical recombination events to the population, thus increasing the resolution of the QTLs. It also widens the represented diversity and can allow access to more causal loci than those segregating in a biparental population. However, some drawbacks of biparental and natural populations still remain. As the number of population

founders is limited, they do not represent the same proportion of the species' diversity than a classical GWAS panel. Moreover, as for linkage mapping population, the creation of a multiparental population requires several generations of crosses.

The two major types of multiparental populations were developed in barley: Multi-parent advanced generation inter-cross (MAGIC) (Sannemann et al., 2015) and two nested association mapping (NAM) (Maurer *et al.*, 2015; Nice *et al.*, 2016; chapter 11) populations. NAM populations are made by crossing multiple inbred lines with a single one and subsequently deriving RILs or DH lines from the progeny (Yu et al., 2008). The common parent genotype normalizes the genetic background, and common-parent specific markers are used to genotype the population. So even as diversity is introduced by the diverse parental lines, the use of a common parent reduces the creation of new haplotypes, since no new haplotypes are recreated between the unique parents. MAGIC populations (reviewed by Huang *et al.*, 2015) imply a greater number of crosses than NAM populations and are thus more labor-intensive to make, but include more diversity. These populations are made by intercrossing several founders (typically eight) and selfing the lines, then using the progeny of those crosses to obtain RILs. Allele frequency is more balanced than in NAM populations and haplotype diversity is higher, but these populations are more time-consuming to develop and cannot be extended to additional founders as NAM populations can.

6 From an interval to the causal gene: from high-resolution mapping to gene cloning

Whether linkage mapping or GWAS is used, the resolution achieved is rarely sufficient to directly identify the causal gene or polymorphism. High-resolution mapping of the locus of interest is most often needed to reduce the region and obtain an interval containing a limited number of candidate genes. However, in the case of a quantitative trait, not all QTLs are suitable for high-resolution mapping. QTLs with small effects or low statistical support should be avoided, as the chance of success in high-resolution mapping is low. High-resolution mapping relies on increasing recombination in a gene interval saturated with markers. Single-gene traits can be mapped at a high resolution in F2 populations. To obtain higher resolution, RILs can be derived specifically from plants recombining in the gene interval (segmental recombinants to obtain so-called segmental RILs). However, for quantitative traits, the locus should first be "mendelized", meaning that a high-resolution mapping population must be developed where the inheritance of the phenotypic variation follow Mendel's law: two distinct phenotypic classes, easily distinguishable, must be obtained. To reach this goal, the number of segregating loci affecting the trait must be reduced by inserting the two alleles at a locus in a common genetic background. Near isogenic lines (NILs) are thus produced. NILs are preferably homozygous lines, often containing a single locus from a donor parent (RILs, DHs, F_2/F_3, or original accessions containing the trait of interest) in the genetic background of a phenotypically distinct recurrent parent. NILs are produced by crossing those two parents, then performing backcrossing and/or selfing. A population is then created by crossing the obtained NIL with the recurrent parent, and high-resolution mapping is performed in this new population.

With the tools of today, saturating a locus interval with markers is usually unproblematic. Most of the time, high-resolution mapping will result in an interval containing several candidate genes. The next step is typically a candidate gene approach. With the availability of an annotated reference genome, retrieving lists of candidate genes is straightforward. If one (or a small number) of those

genes stands out for its predicted function, it can be prioritized for further analysis. However, this simple case may not occur. It is possible that the causal gene has no obvious predicted function. Another possible reason is that phenotypic traits can be due to presence/absence variations (PAV) (Feuk et al., 2006) and the sequencing of the interval in parents may still be needed to find the right candidate gene. With the decreasing cost of *de novo* assemblies, we will, in a very near future, see even several high quality genome sequences published per species. Already initiated in maize (Lu et al., 2015; Hirsch et al., 2016; Yang et al., 2017; Sun et al., 2018; Springer et al., 2018), this development empowers us with possibilities to compare the structure and genes present in mapping intervals in a greater proportion of the diversity, and thus decreasing the risk of missing the causal gene that can be absent in one genome (Monat et al., 2018; Worley et al., 2017). To identify the polymorphisms that can explain the phenotypic differences between parents, a preferred candidate gene (or the complete set of genes in the interval) is usually resequenced. Since the trait can also be due to differential gene expression, it is possible to investigate gene expression differences by using microarray (Close et al., 2004), qPCR or RNA-seq (Costa-Silva et al., 2017).

Such approaches allow a researcher to identify a reduced number of candidate causal genes for further validation. A common gene validation method in barley is mutant analysis to identify the effects of mutations that impair or alter a gene's function. This approach typically relies on methods like RNA interference, chemical or physical mutagenesis or T-DNA insertion. In the case of chemical and physical mutagenesis, the mutation is not targeted and screening of the mutants can be performed by targeting induced local lesions in genomes (TILLING, later detailed). Another approach is the use of genetic transformation to either overexpress the candidate gene in a plant without the trait of interest, or to supplement a mutant. Contemporary genome-editing technologies like TALENS (Gurushidze et al., 2014; Wendt et al., 2013) and CRIPR-Cas9 (Lawrenson et al., 2015) are becoming a method of choice to generate mutants in barley at specific loci (Lawrenson et al., 2015; Holme et al., 2017; Kapusi et al., 2017; Kumar et al., 2018). However, these methods should allow us to achieve a larger spectrum of genome editing in a near future, from complete gene insertion or knockout to single base replacement (Bortesi and Fischer, 2015), which would allow to unravel the role of each candidate polymorphism in the trait by single nucleotide edition.

7 Emerging mapping strategies: fast NGS-enabled technologies

While the standard methods described so far are very efficient, even faster methods were introduced in the genomics era. The classic quantitative genetic method to identify rapidly a gene or QTL interval is by bulk segregant analysis (BSA) (Giovannoni et al., 1991; Michelmore et al., 1991). It is based on the genotyping of individuals selected from the two tails of the phenotypic distribution of a mapping population or of a diversity set (Figure 2). Plants from both ends of the distribution can be genotyped individually, but further economic savings can be made by genotyping pooled DNA from groups of individuals with similar phenotypes. The frequency or the strength of signal at markers near the causal locus shows a significant deviation between the two pools, allowing identification of the genes or QTLs loci, with a very limited genotyping effort. BSA has been used for the first time in the early 90s in barley (Barua et al., 1993) but, while early applications were confounded by insufficient marker density resulting in a high rate of false positives, state-of-the-art high-throughput genotyping methods have alleviated this problem. The accurate phenotyping of the entire original population and the real cost and labor are the only remaining limitations (Gallais et al., 2007; Sun et al., 2010).

Figure 2: Schematic representation of Bulk Segregant Analysis (BSA). A population is generated by crossing two parents with distinct phenotypes. The offspring are phenotyped and two bulks, comprising the individuals presenting the most extreme phenotypes of each class, are created. The two bulks are sequenced and their genotypes are graphed along the chromosomes. Candidate intervals are defined as loci where the two bulks show a divergent genotype.

Another fruitful method is the use of mutants exhibiting a trait of interest, or the loss of a trait of interest, to find the mutation responsible for the trait in a reverse genetics approach. TILLING (McCallum *et al.*, 2000; see chapter 10) combines chemical mutagenesis with high-throughput genome-wide screening for point mutations in genes of interest. The original TILLING method used denaturing HPLC for mutation discovery. For higher throughput, a protocol using Li-Cor DNA analyzer was soon developed (Till et al., 2003). Until recently, barley TILLING populations were mostly characterized either by dHPLC (Caldwell et al., 2004) or Li-Cor (Gottwald et al., 2009; Kurowska et al., 2012; Lababidi et al., 2009; Szarejko et al., 2017; Talamè et al., 2008). However, both still involve inconvenient steps, such as acrylamide gel preparation and amplicon purification, and require labeled primers that can impact mutation detection by reducing PCR efficiency. For these reasons, protocol using Fragment Analyzer™ (Advanced analytical technologies) were developed (Kang et al., 2018; Szurman-Zubrzycka et al., 2018). A further future direction of TILLING in barley is NGS-sequencing-based approaches. Such a protocol was developed by Tsai et al. (2011), consisting in sequencing different PCR target amplicons in DNA pools. This method is still expensive and so far restricted to species where the mutation density is high, like wheat (Krasileva et al., 2017). With decreasing costs of sequencing, it is probable that TILLING will move to detection of mutations by sequencing in a near future.

Combining sequencing and mutant screening is the goal of mapping-by-sequencing, following SHOREmap (Schneeberger et al., 2009) or similar analysis like MutMap (Abe et al., 2012). The idea is to combine EMS mutagenesis with whole genome sequencing in an approach similar to BSA. A mutant is generated, most often by EMS. As EMS mutation causes several mutations in the genome, the simple sequencing of one mutant is not sufficient to identify the causal mutation; the mutant must be crossed with the non-mutated parent and a BSA analysis is performed. Complete sequencing of the genome of the mutants allows to retrieve all polymorphisms (Abe et al., 2012; Schneeberger et al., 2009). Since the barley genome is large and complex, less expensive methods using genome complexity reduction were developed.

Mapping-by-sequencing was developed and performed successfully in barley (Jost et al., 2016; Mascher et al., 2014; Pankin et al., 2014). After phenotyping, plants with the mutant phenotypes and those with wild type phenotypes were separated into two pools. DNAs from both pools were subjected to exome capture (Mascher et al., 2013) as a complexity reduction method and subsequently sequenced on a Illumina platform. The obtained reads were mapped against the reference genome and SNPs are detected. Allele frequency in the two pools was then mapped along the genome and a clear imbalance of allele frequency between the two pools could be observed. This method allows precise mapping of a gene with one sixth of the sequencing load needed for similar resultion using WGS sequencing. However, the barley exome capture assay published in Mascher et al. (2013) was estimated to capture around 86 % of the high-confidence exons and no exome capture assay can capture 100 % of the genes, thus there is always an increased risk of missing the target gene by this approach.

For the specific case of pathogen resistance, Steuernagel et al. (2016) described a method combining sequencing of captured targets in the genome ,and mutagenesis to clone genes. Successfully applied in bread wheat, this method, called MutRenSeq, aimed at cloning dominant pathogen resistance genes. Those genes are most frequently represented by NLR genes, whose structure is highly conserved in the genome and can be enriched in sequencing libraries and specifically sequenced in a capture assay called RenSeq (Jupe et al., 2013). MutRenSeq consists in producing loss of resistance mutants by EMS mutagenesis. Independent susceptible mutant lines are then sequenced using the RenSeq protocol (Figure 3). The NLR genes are assembled and aligned to those of the resistant parent. If a gene harbors polymorphisms in all susceptible mutant lines, it is identified as the candidate gene for resistance. The application of RenSeq reduces the amount of data to be sequenced to less than 0.1% of the genome. However, even in the case of dominant resistance, the causal gene may belong to a different gene family. Thus, there is also in this case an elevated risk of missing the target gene.

To alleviate the risk of missing the target gene, either because it is missing from the capture but also in case it is due to PAV or large structural variation that cannot be detected by conventional mapping to a reference genome, methods using local *de novo* assembly were designed. One of these methods is MutMap-Gap that combines the sequencing reads mapped in the gene interval with the unmapped reads to perform a small-scale *de novo* assembly (Takagi et al., 2013). But while this method is efficient in a small genome like rice, the sequencing load to achieve a sufficient coverage in barley would be very high and a complexity reduction method is required. Taking advantage of chromosome flow-sorting technology and efficient *de novo* assembly methods (ChromSeq) (The International Wheat Genome Sequencing Consortium, 2014), Sánchez-Martín *et al.* (2016) proposed a method called MutChromSeq. It consists in generating EMS mutants and screening those for loss of the desirable trait, then flow-sorting the chromosomes of the interesting mutants to select the chromosome that contains the locus of interest (Figure 3). Then, this single chromosome is sequenced and assembled *de novo*. The mutation overlap in independent mutants allows rapid and precise identification of the gene underlying the trait. While chromosome flow-sorting is generally complex, it is well optimized for barley (Doležel et al., 2012), reduces the cost of sequencing, and achieves a greater depth of sequencing to perform a better assembly. These methods are very efficient for genes producing strong phenotypes, but more quantitative traits still elude such approaches. Moreover, they depend on the identification of loss-of-function mutants, which can be difficult to obtain. Targeted chromosome-based cloning via long range assembly (TACCA) (Thind et al., 2017) is designed to avoid these flaws. Like MutChromSeq, it is based on sequencing of the flow-sorted candidate chromosome and its assembly, which needs to reach high contiguity. To achieve it, Dovetail Chicago(TM) sequencing libraries are prepared and sequenced in addition to the classic Illumina libraries to perform long range scaffolding with state-of-the-art assembly pipelines (Putnam et al., 2016). The resulting chromosome sequence is used to resolve structural variation in the gene interval and to design additional markers. While slower than MutChromSeq, this method is faster and more precise than a classical high-resolution mapping and can be applied to all kinds of traits.

Figure 3: Schematic representation of two fast-enabled gene mapping methods. Seeds from the accession presenting the trait of interest are mutagenized, usually by EMS, and the mutants are phenotyped. A few mutants presenting a loss of function for this trait are selected. MutRenSeq consists in capturing the NLR genes in these mutants and sequencing them in a method called RenSeq. NLR sequences are then *de novo* assembled and aligned to the susceptible reference. The

candidate genes are the NLRs presenting a mutation in all the loss-of-function mutants sequenced. MutChromSeq and TACCA consists in performing chromosome sorting on the loss-of-function mutants, as well as the wild type parent, to select the chromosome harboring the locus coding for the trait of interest. These sorted chromosomes are sequenced, *de novo* assembled and aligned. The candidate genes are the ones showing a mutation in all loss-of-function mutants.

8 Conservation of barley germplasm

The genus *Hordeum* comprises cultivated barley *H. vulgare* spp. *vulgare*, its wild subspecies *H. vulgare* spp. *spontaneum,* and over 30 wild relatives constituting the second and the tertiary gene pools that can be a useful source of diversity (Blattner, 2009). Because of its economic importance, barley germplasm has been collected and stored for about a century and now ranks third in terms of the number of accessions kept in *ex situ* genebanks after wheat and rice (Commission on genetic ressources for food and agriculture; FAO, 2010). Knüpffer (2009) estimated that over 450,000 *Hordeum* accessions are kept in *ex situ* collections distributed over 204 genebanks, including almost 300,000 accessions from *H. vulgare* spp. *vulgare* and around 32,000 of *H. vulgare* spp. *spontaneum*. Among these genebanks, seven currently hold more than 20,000 accessions: the PGRC in Canada (http://www.agr.gc.ca/pgrc-rpc), the NSGC in the US (http://www.ars-grin.gov/npgs), the EMBRAPA CENARGEN in Brazil (www.embrapa.br/en/recursos-geneticos-e-biotecnologia), the ICARDA in Syria (www.icarda.org/research-sub/biodiversity-and-its-utilization), the John Innes Center in the UK (www.jic.ac.uk/research/germplasm-resources-unit/), the IPK Gatersleben in Germany (www.ipk-gatersleben.de/en/gbisipk-gaterslebendegbis-i/), and the VIR in Russia (www.vir.nw.ru/). These resources are available to any researcher, and allow the mining of new traits, genes, and alleles.

From those genebanks, panels of germplasm and core collections are identified to represent the largest diversity possible from a size-limited subsample of accessions. Core collections allow the screening of a species' diversity with minimal effort. The International Barley Core Collection (BCC) is constituted of about 1500 accessions, including some *H. vulgare* spp. *spontaneum* and some wild relatives (Knüpffer and van Hintum, 2003). It was characterized genetically and phenotypically for some traits. Other core collections include the NSGC Barley Core (Muñoz-Amatriaín et al., 2014) with 1860 accessions or the Landrace Collection LRC1485 that consists of 1485 spring barley landraces selected specifically for the study of adaptation to climate (Pasam et al., 2014). Some of these panels are also constituted to represent the diversity in the wild relatives like the Wild Barley Diversity Collection that consist in 318 *H. vulgare* spp. *spontaneum* accessions (Steffenson et al., 2007) or the Barley1K collection that comprises 1020 wild barleys sampled in 75 sites in Israel (Hübner et al., 2009). All these collections are genetically characterized, for example by the 9k iSelect array in the case of the NSGC Barley Core (Comadran et al., 2012), as well as phenotypically for some traits, and have been used for GWAS or for germplasm mining.

But, as carefully as a core collection is constructed, it cannot contain all the diversity contained in a species, therefore limiting their use for discovery of very rare alleles. IPK Gatersleben recently completed GBS sequencing of its entire barley collection (Milner et al., Unpublished data). The related webtool (http://bridge.ipk-gatersleben.de) permits to mine the germplasm of the barley collection and design subsets of it based on criteria like genetic or geographic diversity, but also to deliver direct access to the SNPs discovered via an intuitive visual interface. This initiative aims at unlocking the collection for allele mining and diversity studies and is already applied to other genebanks, expanding

the genomic information of genetic resources and allowing association mapping to be performed on a subset of a genebank without additional genotyping.

9 Genetic and genomic resources of barley

Characterization of barley germplasm has been ongoing for a long time and resources are stored in online databases. GrainGenes (https://wheat.pw.usda.gov/GG3/barley_blvd) (Carollo et al., 2005) is a database harboring molecular and phenotypic information of Triticeae and Avena, including genetic maps, markers and germplasm information. Some databases like PLEXdb (http://www.plexdb.org/) (Wise et al., 2007) or GENEVESTIGATOR (https://genevestigator.com/gv/doc/content.jsp) provide access to the pattern of expression of the genes represented on the Affymetrix microarray. RNA-Seq data from Morex cultivar is also available at morexGenes (https://ics.hutton.ac.uk/morexGenes) as well as a barley epigenome browser (https://ics.hutton.ac.uk/barley-epigenome).

The systematic sequencing of the barley genome (International Barley Genome Sequencing Consortium, 2012; Mascher et al., 2017; Mayer et al., 2011) ran in parallel with the development of new tools to access the data. BARLEX (http://barlex.barleysequence.org) (Colmsee et al., 2015) includes the different types of data produced for the barley sequence assembly (Mascher et al., 2017). Centered on the minimum tilling path of the sequenced BACs, it gives access to information such as the number of contigs and sequences of each individual BAC assembly, tables of published annotated genes, mapping of the 9k iSelect markers on the genomes, and a link to a BLAST server (http://webblast.ipk-gatersleben.de/barley_ibsc/) allowing for homology searches against the different published assemblies as well as other databases like full-length cDNA, ESTs, or regions sequenced by exome capture.

A second way to access the genome assembly data is through the genome browser displayed by the common tool of Gramene (http://www.gramene.org) (Tello-Ruiz et al., 2016) and Ensembl Plants (http://plants.ensembl.org) (Kersey et al., 2016) where annotation of genes, as well as full cDNA and transcriptomic data, can be visualized along the genome. These web tools also allow comparative analysis between genomes to be performed. A third useful database for exploring barley genomic data is PGSB PlantsDB (http://pgsb.helmholtz-muenchen.de/plant/barley/index.jsp) (Spannagl et al., 2016). It contains visualization of the different versions of sequence assembly, information on annotated genes as well as synteny comparison with the genomes of *Brachypodium distachyon* and *Oryza sativa.*

10 Case Study: From *rym4* to *rym11*, illustration of paradigm shift in disease resistance mapping and cloning

To illustrate recent advances in positional cloning, we compared the mapping of two resistance genes, performed in the same labs. The first is the *rym4/rym5* locus that was cloned in 2005 (Stein et al., 2005). The second is *rym11*, cloned almost ten years later, in 2014 (Yang et al., 2014b). Both genes confer complete recessive resistance to soil-borne Bymoviruses BaMMV and BaYMV and encode for susceptibility factors.

The *rym4* gene was introgressed from landrace ‘Ragusa’ into commercial German barley varieties as early as the 1980s but its genetic basis was unknown. This recessive resistance gene was proven to be linked or allelic to *Rym1* by allelic test in a cross with *Rym1* variety Mokusekko 3 (Friedt et al., 1987). Crosses between the *rym4* variety Franka and barley accessions exhibiting a strong dominant

phenotype mapped to a chromosome (genetic marker stocks) proved that *rym4* was neither on chromosomes 4H, 1H nor 5H. (Kaiser and Friedt, 1992, 1989), but placed *rym4* distal on the long arm of chromosome 3H by segregation analysis in crosses between the *rym4* 'Ogra' and 'Sonate' varieties and trisomic lines (lines trisomic for specific chromosomes) and telotrisomic lines. From these early attempts, successive innovations in molecular markers allowed the precise characterization of the genetic interval. In the 1990s, several studies used RFLP, isozymes and RAPD markers and mapped *rym4* and *rym5* in the same interval at the distal end of chromosome 3HL (Graner et al., 1999, 1995; Graner and Bauer, 1993; Konishi et al., 1997; Ordon et al., 1995). Despite the increase in numbers of marker types and of population sizes, the initial interval of 2.4 cM (Graner and Bauer, 1993) was not significantly reduced during this decade. The first high-resolution mapping of the locus *rym4*/*rym5* (Pellio et al., 2005) took advantage of advances in molecular markers to screen large populations: 3,884 F_2-plants for *rym5*, and 1,040 F_2 individuals for *rym4* mapping. DNA bulks from individuals of these DH populations were used for AFLP, RAPD and RFLP marker saturation of the regions and RILs were derived from recombinants between the common flanking markers of *rym4* and *rym5*. The new markers were converted into STSs and used to genotype the RILs, reducing *rym4* and *rym5* intervals to 0.05 cM and 0.013 cM, eventually leading to the cloning of the gene (Stein et al., 2005). The Morex BAC library (Yu et al., 2000) was screened with the markers of the *rym4/rym5* region, and a 650 kb physical map was constructed. The six BAC clones cosegregating with resistance were sequenced to full length and annotated (Wicker et al., 2005), and two open reading frames (ORFs) were found. One of them, *Hv-eIF4E*, was homologous to genes involved in recessive *Potyvirus* resistance in dicotyledonous species and was sequenced in 56 barley accessions known to carry either *rym4*, *rym5*, an unknown source of resistance, or no resistance. This sequencing revealed several non-synonymous polymorphisms in *rym4* and *rym5* accessions and this gene was confirmed by complementation via *Agrobacterium*-mediated transformation of a *rym4* NIL with either the full length cDNA or the genomic DNA of a susceptible accession, thus inducing susceptibility. Kanyuka et al. (2005), performed sequencing of RT-PCR products of different variants and evidenced that *rym6* is another natural allele of *Hv-eIF4E.* They also carried out TILLING on the gene and retrieved one mutant allele, with which resistance segregates. Additional allele mining was later performed, identifying several other alleles of resistance at this gene (Yang et al., 2017, 2014a).

In contrast, *rym11* mapping was comparatively fast. The preliminary mapping of *rym11* was performed on two populations of 48 F_2 and 101 BC_1F_2, respectively (Bauer et al., 1997). The authors performed BSA with RFLPs, RAPDs and one SSR marker, which localized the gene to within a large interval of 16.4 cM, close to the centromeric region of chromosome 4HL. The first fine-mapping study (Nissan-Azzouz et al., 2005) analyzed three DH populations of 57, 191 (IPK1) and 161 (IPK2) DH lines, respectively. The authors successively performed an initial mapping with RFLP markers, two BSA analyses with RAPD and AFLP markers respectively, and a more complete mapping with SSR, RAPD and AFLP markers. Based on the results, *rym11* was mapped in a 3.7 cM interval in IPK1 and a 10.7 cM interval in IPK2 population. Lüpken et al. (2013) used a genomics-informed approach to resolve the interval in a population comprising 5,102 F2 plants. To carry out the mapping, they used publically available SSR and SNP markers and designed additional SNP and STS markers with the information available in EST databases, in the barley genome zipper (Mayer et al., 2011), as well as in the WGS sequence assembly of Morex (International Barley Genome Sequencing Consortium, 2012). Polymorphisms were revealed either on gels or by pyrosequencing, leading to a final interval of 0.074 cM. The gene *rym11* was eventually isolated by chromosome walking (Yang et al., 2014b). Barley BAC

clones (Ariyadasa et al., 2014; Schulte et al., 2011), sequenced in conjunction with the barley genome sequencing project (Mascher et al., 2017) were identified by sequence comparison to the flanking markers. Two overlapping BAC contigs were identified, resulting in a physical contig covering the complete genetic interval. Based on the available BAC sequence data, new markers were designed to reduce the interval to 0.0196 cM, representing 1.25 Mbp, using the same 5,102 F_2 population previously explored by Lüpken et al. (2013). Annotation of this interval revealed four open reading frames (ORFs). These ORFs were resequenced, and only one gene exhibited polymorphism between susceptible and resistant genotypes. This gene encoded a putative protein disulfide isomerase like 5-1 (HvPDIL5-1), and the resistant plants' allele contained a 1.3 kb deletion in its promoter and first exons. It was validated as the resistance gene by TILLING in an EMS-induced mutant population and by complementation via *Agobacterium*-mediated transformation of the resistant genotype with the cDNA of the gene from a susceptible accession. Moreover, the gene *HvPDIL5-1* was sequenced in 1,732 diverse barley accessions, both domesticated (*H. vulgare* ssp. *vulgare*) and wild (*H. vulgare* ssp. *spontaneum*), and identified three more natural haplotypes conferring resistance, all harboring a premature stop codon in *HvPDI5-1*. This allele mining was later completed in a larger collection (Yang et al., 2017).

These studies illustrate how developments in molecular marker technology have revolutionized gene mapping studies, allowing the genotyping of large populations more easily and, by increasing the number of useful markers available, allowing the discovery of intervals smaller than 1 cM. Increased availability of sequence data represented a second game-changer. It enabled for instance the design of numerous markers in the interval of *rym11*, sufficient to achieve a mapping resolution below 0.1 cM. It has also accelerated the acquisition of physical maps. The tedious chromosome walking carried out for *rym4* included the screening of a BAC library and the sequencing of the identified BACs, whereas for *rym11* the sequences were already available and BAC clones screening was performed *in silico*. Moreover, TILLING and allele mining for both loci provided a fast way of validation as well as alternative alleles that could be of use for breeding. The *rym11* mapping is now already five years old and new methods of sequencing have rendered gene mapping studies even faster and more precise (Jost et al., 2016; Mascher et al., 2014).

11 Conclusion and future trends

Advances in sequencing technology and the availability of the barley reference genome have underpinned genomics-informed barley gene mapping and cloning. Genotyping is progressively faster, easier and relatively more cost-efficient. New NGS-based gene mapping methods are in place and more are appearing. A paradigm-changer for gene mapping in the coming years will be the spread of *de novo* assemblies of complete barley genomes or flow-sorted chromosomes. More reference-quality *de novo* assemblies of different barley haplotypes are likely to come as a result of pan-genome analysis (Hurgobin and Edwards, 2017). Already initiated (https://www.pflanzenforschung.de/de/plant-2030/fachinformationen/projektdatenbank/strukturelle-genomvariation-haplotypendiversitaumlt-und-419), which will continue to unlock the diversity of barley, including structural changes, so far unreachable with a single reference, and increasing the efficiency of gene cloning.

In addition to classical gene and QTL mapping, the future will probably see an increase in expression QTL (eQTL) (Damerval et al., 1994) analysis. Barley gene expression is affected by complex regulation.

More than half of the genes have been found to be differently regulated between samples, and more than two thirds have been shown to exhibit alternative splicing (International Barley Genome Sequencing Consortium, 2012). Moreover, quantitative traits are often regulated by the differential expression of genes. In eQTL analysis, gene expression is quantified, and related to the phenotype. Whole genome eQTL analysis has been performed on barley (Potokina et al., 2008) using RNA binding on an Affimetrix chip. However, it is now possible to perform it by RNA-seq, achieving a better throughput. Moreover, the methylome can also be sequenced with next generation sequencing. Called whole genome bisulfite-sequencing (BS-seq), this method is based on sodium-bisulfite treatment, that converts unmethylated cytosine of the genomic DNA to uracil, followed by whole genome sequencing. With this method, a single base methylome map of *Arabidopsis thaliana* was generated (Lister et al., 2008). It was also performed recently on barley Morex accession (Wicker et al., 2017), and could thus be applied to trait mapping in a near future.

However, despite the huge progress made in 30 years, efficient gene mapping is still facing several bottlenecks. Time of generation during population construction, or to obtain progenies for phenotyping, is slowing down a lot of projects. Watson et al. (2018) developed 'speed breeding' to shorten the breeding cycle. This is achieved by increasing daily light exposure and harvesting the plants at early growth stages. By this process, the number of barley generations in a year was doubled, from three to six. A low-cost version of 'speed-breeding' makes this protocol available to the whole community and will probably be intensively used in the upcoming years (Ghosh et al., 2018). Another challenge faced by trait mapping is computing power. The amount of sequencing data available grows as the price of sequencing decreases. Previous data analysis systems are no longer sufficient to process the data generated (for review see Yin et al., 2017), and storage quickly becomes a limiting factor in a lot of labs. To keep up with modern analysis and data requirements, labs must invest in appropriate bioinformatics platforms and trained staff.

As genotyping methods develop and the cost of sequencing decreases, genotyping ceases to be a primary bottleneck in trait genetics. However, whether it is for GWAS or for mutant screening, many of plants need to be phenotyped and this task can be arduous. Therefore, phenotyping has become a more primary concern, particularly in the case of quantitative yield and stress response traits that necessitate field phenotyping, and are strongly influenced by GxE effects. Progress in imaging using sensors and drones (for review Tardieu et al., 2017) are beginning to alleviate this problem, but development of so-called phenomics is still in its infancy and remains very costly. However, even if limited in the traits to which it can be applied, phenomics will become a major part of trait mapping in the future, allowing mapping of some complex traits currently far out of reach.

12 Where to look for further information

Further reading

- Bazakos et al. (2017): Annual review of plant biology: a good review on quantitative genetics methods. From phenotyping, to populations and QTL analysis.
- Grover and Sharma (2016): a good review on the different type of markers and their compared qualities.
- Jiao and Schneeberger (2017): a review summarizing the third generation sequencing that render assembly of barley possible.

- Rifkin (2012): a very complete book on QTL mapping, from population construction to marker selection and statistical analysis of the data
- Stein and Muehlbauer (2018): summarizes the state-of-the-art in barley genome analysis

Key journals/conferences

- PAG (Plant and Animal Genome): the biggest conference on genetics and genomics. Held in January every year in San Diego, USA.
- International Barley Genetics symposium (IBSG): symposium on barley genetics held every four years

- Theoretical and applied genetics (TAG)
- Molecular breeding
- Plant breeding
- Molecular Plants
- Plant Journal
- Plant Physiology
- PNAS
- Plant Cell
- Nature Genetics

Major international research projects

- International Barley Sequencing Consortium (IBSC)

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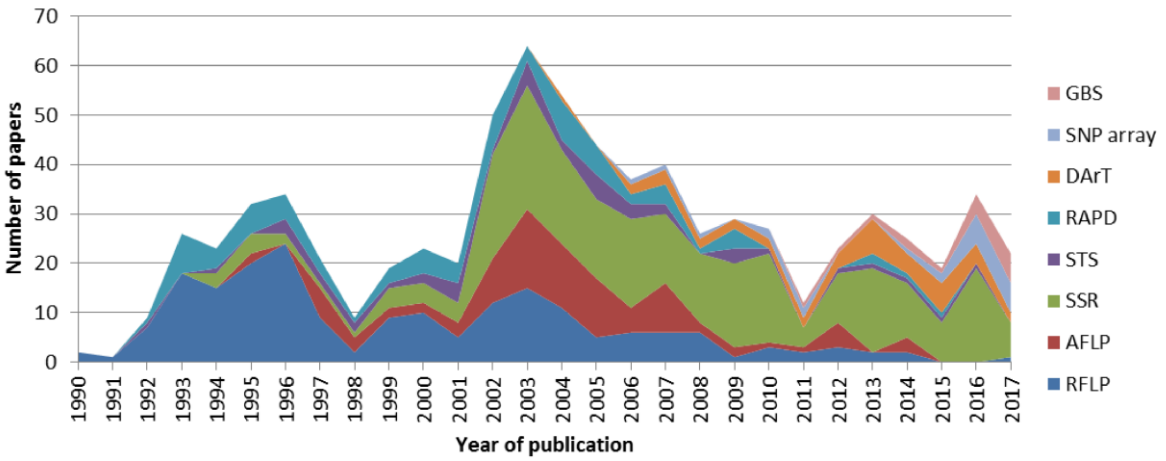
70
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Number of papers
1990
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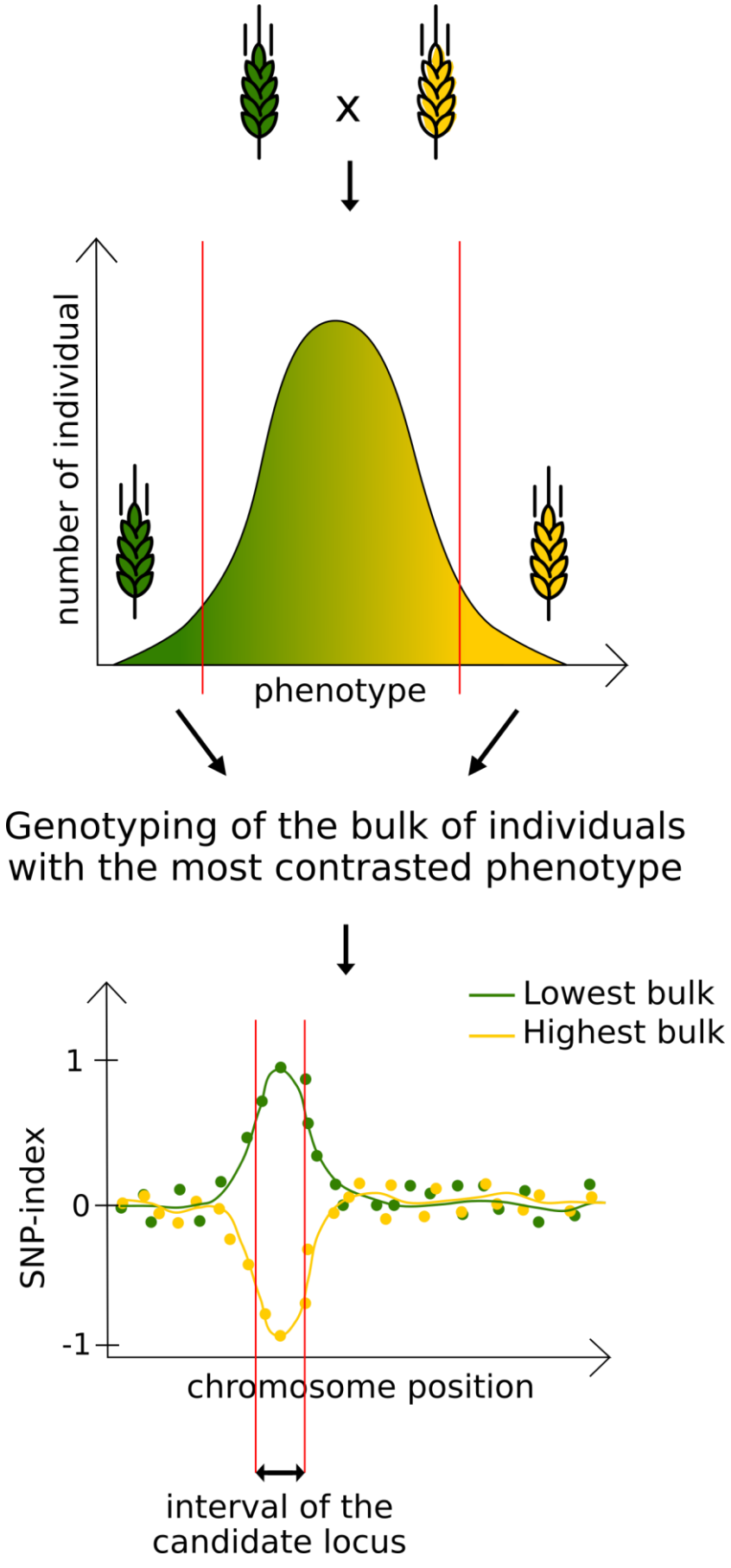
x
number of individual
phenotype
Genotyping of the bulk of individuals
with the most contrasted phenotype
Lowest bulk
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1
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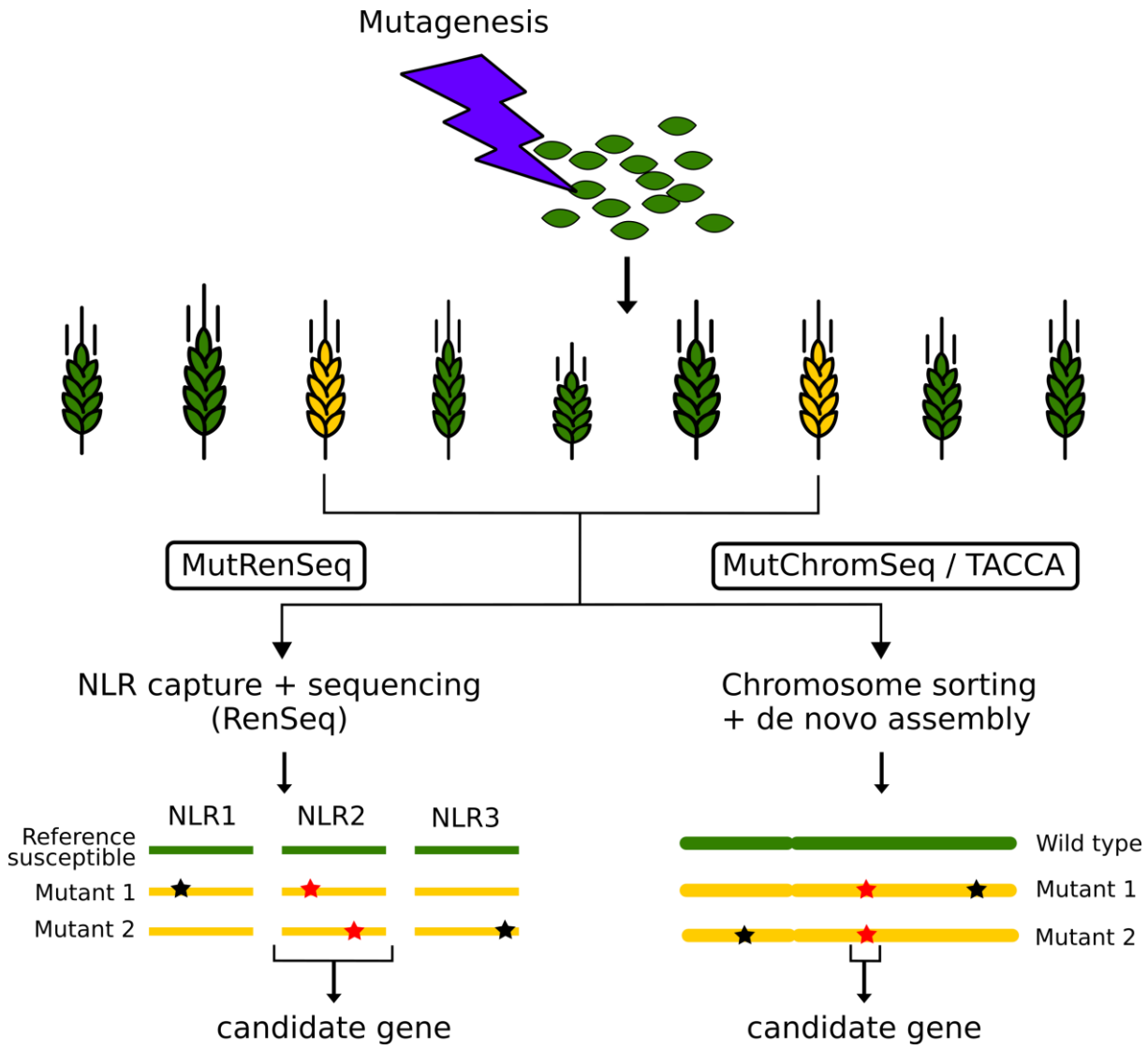
SNP-index
chromosome position
interval of the
candidate locus


Mutagenesis
MutRenSeq
MutChromSeq / TACCA
NLR capture + sequencing
(RenSeq)
Chromosome sorting
+ de novo assembly
NLR1
NLR2
NLR3
Reference
susceptible
Mutant 1
Mutant 2
Wild type
Mutant 1
Mutant 2
candidate gene
candidate gene

Marker	RFLP	AFLP	RAPD	SSR	DArT	SNP array	GBS
Co-dominant/Dominant	Co-dominant	Dominant	Dominant	Co-dominant	Dominant	Co-dominant	Co-dominant
Genome abundance	High	Very high	Very high	Medium	Very high	Very high	Very high
Reproducibility	High	High	Low	High	High	High	High
Number of marker scored per assay	Low	Moderate	Low	Low	Moderate	High	High
Technical requirement	High	Moderate	Low	Moderate	High	Low	Moderate
Cost per data point	High	Moderate	Low	Low	Very low	Low	Very low
Automation	Difficult	Moderate	Difficult	Moderate	Easy	Easy	Easy
Prior sequence knowledge	No	No	No	Yes	No	Yes	Preferable
Marker development	Fast	Moderate	Long	Long	Long	Long	Moderate
Ease of analysis	Difficult	Difficult	Difficult	Easy	Easy	Easy	Easy
Quantity of DNA required (µg)	10000	500 to 1000	20	50	50 to 100	200	20
Equipment cost	Moderate	Moderate	Low	Low	High	High	High
Throughput	Low	Moderate	Moderate	Moderate	High	High	High

Table 1: Characteristics of the most used markers in barley, currently or in the past.