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Review

Biotechnological and Digital Revolution for Climate-Smart Plant Breeding

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Abstract: Climate change, associated with global warming, extreme weather events, and increasing incidence of weeds, pests and pathogens, is strongly influencing major cropping systems. In this challenging scenario, miscellaneous strategies are needed to expedite the rate of genetic gains with the purpose of developing novel varieties. Large plant breeding populations, efficient high-throughput technologies, big data management tools, and downstream biotechnology and molecular techniques are the pillars on which next generation breeding is based. In this review, we describe the toolbox the breeder has to face the challenges imposed by climate change, remark on the key role bioinformatics plays in the analysis and interpretation of big "omics" data, and acknowledge all the benefits that have been introduced into breeding strategies with the biotechnological and digital revolution.

Keywords: climate change; mapping populations; genetic resources; mutation breeding; genome editing; new plant breeding techniques; "omics" data; bioinformatics

1. Climate Change is Increasing Pressure on Crop Breeding

Climate change is strongly influencing agricultural production and cultivation practices of all major crops with various and heterogeneous effects, which critically depend on geographical areas [1]. The climate variables that directly affect agricultural production are the rapid growth in mean temperatures and the increasing frequency and magnitude of extreme weather events [2].

Water deficit is a growth- and yield-limiting factor for crops worldwide [3]. It has been reported that water scarcity deeply influences flowering, pollination, and grain-filling of most grain crops; on the other hand, abundant rainfalls may have a positive impact on yield and end-use quality, but they may damage plants because of higher relative humidity, which predisposes plants to the outbreak of diseases [4]. Drought also has a major impact on crop yield; however, it has been demonstrated that the severity of the stress depends on the phenological status of the plant [5,6].

Impact of extreme heat waves has been analyzed in wheat [7,8], rice [9], maize [10], and soybean [11]. It has been noted that an increase of 1 °C of seasonal temperatures determines a decrease in yield ranging from 7.4% in maize to 3.1% in soybean [12].

The increase of atmospheric CO_2 has conflicting effects on crops: On one hand, it determines an increase in plant photosynthesis and growth; on the other hand, it negatively affects the nutritional quality of crops as well as their health status [13].

As an example, an increase in barley yellow dwarf virus infections has been observed in wheat under elevated CO_2 levels [14].

Breeding crop varieties for environmental stresses is a slow and challenging process, as the effects of stresses on crops are variable and complex especially when crops are exposed to multiple stresses [4,15,16]. Although various information is available on plant response to a single stress factor, much less is the knowledge on the response mechanisms of crops when exposed to a combination of biotic and abiotic stresses (i.e., simultaneous stresses). Clearly, plant response depends on the combination of specific stresses, on the intensity of each stress, and on the plant developmental stage [17]. Studies demonstrated that plant stress and defense responses are controlled by different, and sometimes conflicting, signaling pathways and that the plant activates specific signaling cascades and metabolic pathways, which differ depending on whether the plant is subjected to individual or multiple stresses [16,18].

Drought, heat stress, and their combination on growth-related traits have been widely investigated. Several studies demonstrated the negative effect of simultaneous high temperature and drought on the growth, development, and reproduction of cereals, thus affecting productivity [19–21]. The combination of drought and salt stress also decreased yield potential in barley [22]. Elevated temperatures combined with drought reduced the performance of grapevine in the Mediterranean basin, but elevated levels of CO_2 could mitigate such damaging effects [23]. Photosynthesis was shown to be sensitive to drought or heat stress. As reported by Feller (2016), the interaction between water scarcity and heat stress affects carbon assimilation in crops. Indeed, leaf temperature, stomatal opening, and water status are strongly interconnected, suggesting a complex regulatory network underlying plant adaptation processes and coordinating gene expression [24].

All these developing threats are leading to an increase in the incidence of weeds, pests, and pathogens, which generally were confined in particular geographical areas. According to predictive models, it is expected that between 2050 and 2100, *Fusarium oxysporum* spp. (Schltdl., 1824) will be the main cause of plant disease in European, Middle Eastern, and North African regions, posing risks to a number of cash crops [25]. At present, they are thriving worldwide because of the simultaneous occurrence of warming temperatures, increasing levels of humidity, CO_2, and ozone levels [26–28]. High temperature and moisture increases the production and germination of propagules and accelerates pathogen growth rates. Elevated temperatures and ozone levels favor infection by necrotropic pathogens. Otherwise, high levels of CO_2, temperature, and drought foster plant colonization by biotrophic pathogens. As an example, *Fusarium* head blight (FHB) and *Septoria tritici* (Desm.) Blotch (STB) diseases in wheat are increasing in China [29], United Kingdom [30], and in several countries of the European Union due to the altered weather patterns [31]. On the other hand, Rejeb et al. [17] reported several examples of cross-tolerance between abiotic and biotic stresses that may induce positive effects and enhanced resistance in plants with significant implications in plant breeding. For instance, drought stress induced an increase of abscisic acid levels with a significant increase of resistance response towards necrotophic fungus *Botrytis cinerea* (Pers., 1794) and *Oidium neolycopersici* (Kiss, 2001), while salt stress reduced *O. neolycopersici* infection [32].

In this challenging scenario, it is clear that we need miscellaneous strategies to develop climate-resilient cultivars and expedite the rate of genetic gains [33]. The understanding of the physiological, genetic, and molecular mechanisms that allow plants to adapt and respond to climate change and the identification of adaptation traits to variable environmental conditions triggered by climate change are among the main objectives of next generation breeding.

Next generation breeding relies on the availability of large plant breeding populations and germplasm collections, efficient high-throughput technologies, big data management tools, and downstream biotechnology and molecular breeding activities. It is allowing and will allow the scientific community to define, in a short time frame, one or more ideotypes suitable to satisfy the breeding demand and to discover superior alleles and haplotypes to be used in breeding programs.

Furthermore, recent advances in genomic knowledge and the increasing availability of information on genes as well as on in vitro regeneration technologies allow the development and use of second-generation biotechnologies, based on cisgenesis and genome editing [34–36], to produce a diverse array of novel value-added products that may be indispensable in addressing future challenges associated with sustainable agriculture.

Genome editing can breathe new life into plant breeding strategies. Indeed, genome editing is opening up novel opportunities for the precise and rapid modification of crops to boost yields and protect against pests, diseases, and abiotic stressors [37–39]. The great potential of the genome editing techniques relies on making crop breeding faster, more precise, and at lower production costs.

In this review, we provide a brief overview of the possibility of exploiting germplasm resources with diverse allelic combinations for genetics research and breeding. Then, we discuss the most recent strategies, cutting-edge technologies, methods, and tools for adapting crops to climate change, and remark on the key role bioinformatics plays in the analysis and interpretation of big "omics" data. Finally, we acknowledge the benefits that have been introduced into breeding strategies through the biotechnological and digital revolution, and we stress the concept that a "new figure" of breeder, with new specializations, is needed.

2. Browsing through the Literature: Trends of the Most Recent and Breakthrough Technologies to Advance Climate-Smart Breeding

In the 1995, the Intergovernmental Panel on Climate Change (IPCC) released the Second Assessment Report on the impact of climate change on the sustainable development of the society. This document has laid the foundations for achieving the international agreement linked to the United Nations Framework Convention on Climate Change, known as the Kyoto Protocol [40]. The report by IPCC describes the assessment of impact, adaptation, and mitigation of climate change with regard to environmental and socio-economic aspects. Following the dissemination of the ideas contained in the document, a growing interest by the scientific community has been observed in the study of the causes and effects of climate change.

The number of published academic papers is a powerful indicator for measuring the development tendencies of certain scientific researches. Literature related to "climate change" is vast and covers several branches of knowledge such as agronomy, molecular biology, physiology, and socio-economic disciplines [41,42]. Janssen et al. [41] and Wang et al. [42] performed a bibliometric analysis to determine qualitative and quantitative changes in the scientific research topics related to the resilience, vulnerability, and adaptation to climate change without taking into consideration the extent to which climate change impacts on plant breeding.

In this review, we analyzed, quite simply, the number of publications in which the most recent, popular, and breakthrough technologies applied to plant breeding were associated or not with climate change. The analysis was based on the information available in the Web of Science database (www.webofknowledge.com), category "Plant science", considering the time interval of 2000–2018. Different keywords (i.e., "plant breeding", "QTL (Quantitative Trait Loci)", "association mapping" and "GWAS (Genome Wide Association Studies)", "genomic selection (GS)" and "GS", "genome editing" and "mutagenesis") and Boolean operators were used to query the database (Figure 1).

The results showed that the largest number of publications was retrieved using "QTL*" as a keyword (Figure 1B), while the least number of publications affects those documents that included "genome editing" as a keyword (Figure 1E). This trend reflects the recent history of technological advances and methodological innovations in plant breeding. QTL mapping, in fact, is the oldest method used in plant breeding to identify genetic variants that influence the magnitude of measurable traits [43]. On the other hand, genome editing techniques have been introduced much more recently to support plant breeding and require the development of specific protocols that widely vary from species to species.

All the technologies taken into consideration showed an upward trend across the years, particularly after 2013. By contrast, mutagenesis with the exclusion of genome editing (Figure 1F) was the only method with a more stable trend across the years. Table 1 reports the top ten list of the most cited scientific articles retrieved by combining, in a single query, all the keywords mentioned above (Figure 1A).

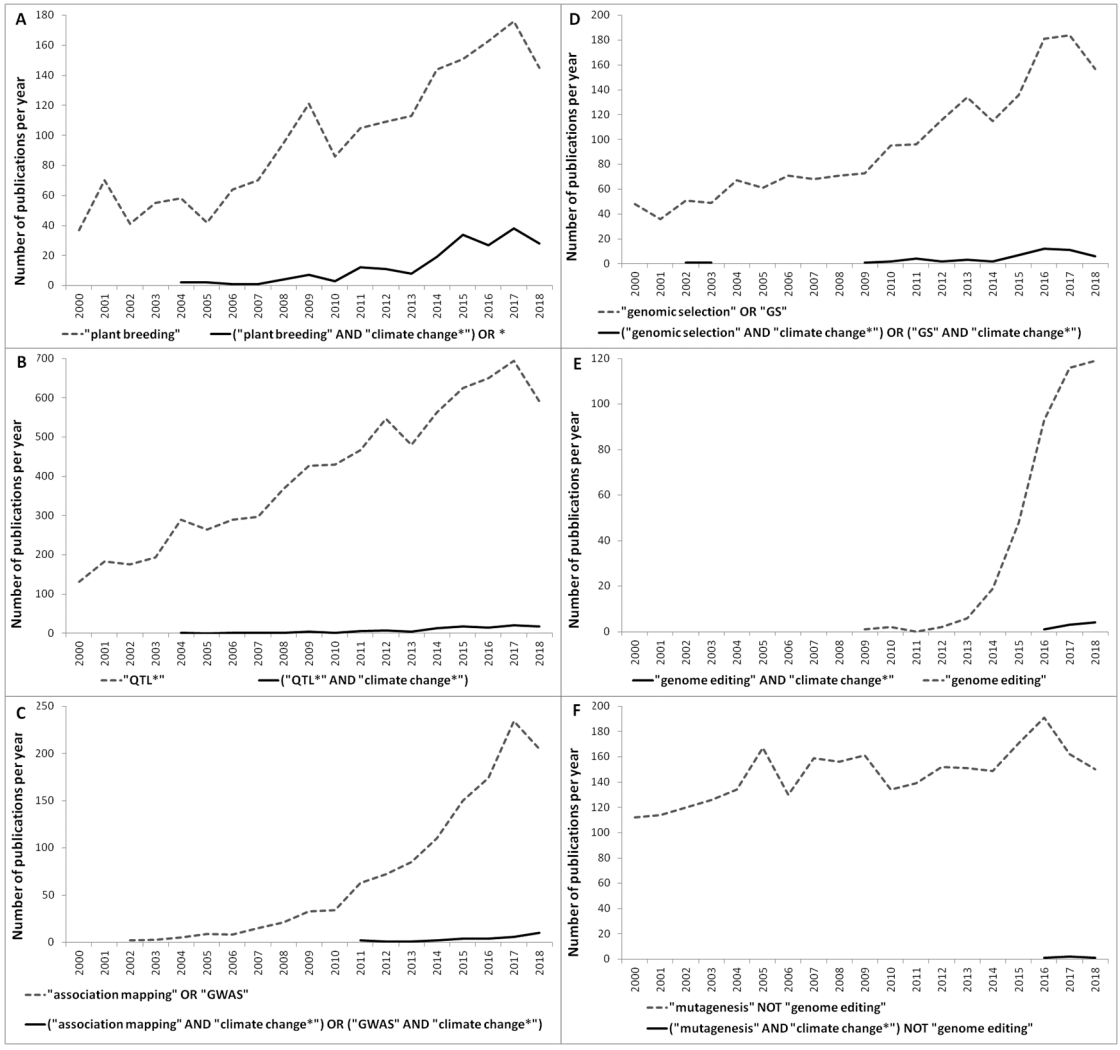

Figure 1. Number of publications in which the most recent and breakthrough technologies applied to plant breeding are associated (—) or not (---) with climate change. (**A**) Keyword: plant breeding; (**B**) keyword: QTL*; (**C**) keywords: association mapping or GWAS; (**D**) keywords: genomic selection or GS (**E**) keyword: genome editing; (**F**) keywords: mutagenesis NOT genome editing. * complete query = (“plant breeding” AND “climate change *”) OR (“association mapping” AND “climate change*”) OR (“gwas” AND “climate change*”) OR (“genomic selection” AND “climate change*”) OR (“mutagenesis” AND “climate change*”) OR (“QTL*” AND “climate change*”).

Table 1. Top ten list of the most cited scientific articles retrieved (up until 11/8/2018) from the Web of Science database, category "Plant science", using the following query: ([("plant breeding") AND ("climate change*")] OR [("association mapping") AND ("climate change*")] OR [("gwas") AND ("climate change*")] OR [("genomic selection") AND ("climate change*")] OR [("mutagenesis") AND ("climate change*")] OR [("QTL*") AND ("climate change*")]).

Reference Title	Journal	Publication Year	Total Citations
Genetic engineering for modern agriculture: challenges and perspectives [44]	*Annual Review of Plant Biology*	2010	356
Breeding for yield potential and stress adaptation in cereals [45]	*Critical Reviewers in Plant Science*	2008	278
Root system architecture: opportunities and constraints for genetic improvement of crops [46]	*Trends in plant science*	2007	255
The ozone component of global change: potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species [47]	*Journal of Integrative Plant Biology*	2009	156
Developments in breeding cereals for organic agriculture [48]	*Euphytica*	2008	147
Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops [49]	*Theoretical and Applied Genetics*	2012	138
Genotyping-by-sequencing for plant breeding and genetics [50]	*Plant Genome*	2012	131
Climate change and diseases of food crops [51]	*Plant Pathology*	2011	99
The stay-green trait [52]	*Journal of Experimental Botany*	2014	82
Quantitative genetic analysis of biomass and wood chemistry of *Populus* under different nitrogen levels [53]	*New Phytologist*	2009	82

3. The Breeder's Toolbox for Facing the Challenges Imposed by Climate Change

3.1. Genetic Resources: A Cornerstone for Competitive Plant Breeding

A deep understanding of adaptive mechanisms to climate changes cannot be separated from detailed knowledge on the genetic background and phenotypic plasticity of crops [54].

Mapping populations are widely used to investigate the relationship between DNA polymorphisms and trait variation [55]. High-resolution trait mapping in crops implies the selection of adequate genetic material from which various germplasm resources can be developed in order to breed climate-resilient crops. The resolution and accuracy of mapping qualitative and quantitative trait loci (the latter referred to as QTLs) depends on the recombination rate and frequency, the effective population size (the larger the population, the higher the frequency of recombination and the higher the QTL resolution), and on trait heritability [56].

In order to dissect the genetic basis of complex traits in crops, geneticists generally use two different types of populations: namely, family-based mapping populations and association mapping populations. As it can be easily understood, the recombination rate and the linkage disequilibrium (LD) decay greatly differ between the two types of populations. Indeed, individuals in family-based mapping populations have accumulated a very low number of recombination events, leading to the presence of blocks of high LD [55].

Bi-parental and multi-parental mapping populations (MPPs) are both family-based mapping populations. Bi-parental mapping populations, classically used for QTL mapping, usually derive from the cross between two contrasting individuals differing for one or more target traits. Their main limitation is that QTL detection depends strongly on the phenotypic diversity of the two parents only and that a few recombination events occur during the development of the population.

Unlike bi-parental mapping populations, whose variation relies on a relatively narrow genetic base, MPPs have been proposed as suitable resources to define the genetic basis of complex traits as they are characterized by high levels of recombination events and larger phenotypic diversity [57–59].

Typically, multi-parent Advanced Generation Inter-Cross (MAGIC) mapping populations are developed by inter-crossing multiple (generally four, eight, or sixteen) parental lines so as to fully exploit their complex pedigree structure [60,61]. Developing a MAGIC population is not trivial, as it requires the identification of founder lines within worldwide germplasm collections, elite cultivars, landraces, and distant relatives with pronounced genetic and phenotypic differences. Generally, the mixing of multiple parents follows different crossing schemes depending on how many founders are taken into account [56]. Although the benefits of working with MAGIC populations are clear, it is also necessary to remark two constraints: (i) the alien introgressions that might occur in the population as a consequence of rearrangements; (ii) the time necessary to develop homozygous individuals derived by advanced inter-crossing. Indeed, it has been estimated that at least eight crop seasons are required to reach at least the S5 generation, which is associated with a residual heterozygosity below 3% [62].

Association mapping populations are developed by collecting hundreds of unrelated individuals among elite and old cultivars, landraces, and wild relatives, which represent an invaluable source of natural genetic variations. Many of these populations include individuals retrieved from different parts of the world and characterized by a wide diversity [63–65]. The great advantage of using association mapping populations relies on the higher allelic richness that is captured and that is essential for high-resolution QTL mapping.

Finally, Nested Association Mapping (NAM) populations have been developed by combining, in a single unified mapping population, the advantages of two different types of populations (i.e., bi-parental mapping populations and association mapping populations) with the purpose of further increasing the precision of QTL mapping [66,67]. Generally, NAM populations derive from the crossing of multiple lines (i.e., diversity donors) with a single "reference" inbreed line, possibly an elite cultivar improved for important agronomic traits and extensively used in breeding programs.

Crosses give rise to multiple bi-parental sub-populations, either as double haploid (DH) lines or as recombinant inbreed lines (RILs), each of which is subjected to self-fertilization for six generations before being genotyped. Finally, parental lines are, in turn, sequenced or genotyped, and the results are overlaid on the recombination blocks previously identified in each sub-population.

3.2. Cutting-Edge Technologies for Breeding Applications

3.2.1. QTL Mapping and Marker-Assisted Selection

The basic idea behind QTL mapping is the identification of DNA molecular markers (such as single nucleotide polymorphisms, SNPs) that correlate with a given trait in a segregant (mapping) population, thus allowing the positioning of QTLs within linkage maps. Quantitative traits can be controlled by a few loci with fairly large effects (i.e., major QTL), or by many loci, each with minute effects (i.e., minor QTLs). Different methods for QTL analysis have been developed so far, and over hundred QTL mapping software have been implemented (for an exhaustive review, see Sehgal et al. [68]).

The advent of new sequencing technologies greatly facilitated the study of genomic variation, as it led to the identification of a large number of DNA polymorphisms, especially SNP markers, at limited cost [69]. The development of dense and ultra-dense linkage maps [68] increased the accuracy of QTL mapping from a region of 10–30 centimorgan (cM) [70] to a region <1 cM on average [71]. As mentioned earlier, a broader genetic diversity (bi-parental vs MAGIC or NAM populations) gives high QTL resolution.

In addition, the availability of high-throughput plant phenomic tools is also of great importance for increasing the potential of QTL mapping [72]. The link between phenotypic traits and genotypic data is essential in explaining the genetic basis of complex traits.

QTLs affecting the phenotypes of interest can be also detected using LD mapping, which takes advantage of historical recombination events within the unobserved pedigree [73].

The resolution of QTL mapping can also be enhanced by combining linkage maps with LD maps [73]. Indeed, the existence of LD implies there are segments of a chromosome in the population which are descended from the same common ancestor. These identical-by-descent (IBD) chromosome segments carry both identical marker haplotypes and identical QTL alleles. This type of QTL mapping is referred to as LDLA (linkage disequilibrium linkage analysis) [73].

Recent studies report the molecular characterization of QTLs together with the identification of DNA polymorphisms underlying important traits, such as resistance to drought in barley and FHB resistance in wheat [74–76]. A large number of QTLs have been identified in cereals for agronomic and physiological traits under heat temperature and water stress conditions. As summarized in the review by Gupta et al. [77], several studies have already been conducted in wheat (*Triticum* L. spp.) using bi-parental interval mapping. Nine major and stable QTLs were detected for coleoptile length, root system, and grain yield, which represent the most relevant traits contributing to seedling emergence, grain yield, and adaption to drought environments [78]. Recently, high-density linkage maps were constructed using SNP markers in bread wheat RILs in order to detect QTLs for flag leaf-related traits, which play a key role in determining yield potential [79].

Once information on SNP-trait associations is available, it can be conveniently used to assist breeding programs. Marker assisted selection can be performed via medium- or high-throughput assays, such as KBioscience's Competitive Allele Specific-PCR SNP genotyping system (KASPar; http://www.lgcgenomics.com/) or high resolution melting (HRM) [80]. In case SNPs are associated with restriction endonuclease sites, they can be converted into cleaved amplified polymorphic sequence (CAPS), easily obtainable without the need of costly equipment [80,81].

3.2.2. Genome-Wide Association Studies and Genomic Selection

Genome-wide association studies investigate marker-trait associations based on the large nucleotide variability present within association mapping populations. The availability of a large number of SNPs is a necessary but not sufficient condition to improve the resolution of marker-trait association, which also strongly depends on the extent of LD decay over physical distance in a population [82]. The mating system of the species, recombination frequency, genetic drift, and the selection process of individuals are the most important factors affecting LD decay [83].

As clearly described by D'Agostino and Tripodi [69], once DNA variation has been captured, it is used to describe the genetic structure of the population under study. Assessment of population stratification (i.e., the presence of a systematic difference in allele frequency spectrum or in principal components between sub-populations) is essential to prevent false positive or negative SNP(s)–trait associations [69,84,85]. In addition, it is essential to have available robust phenotypic data for each individual in the population in such a way that significant genotype–phenotype associations can be scored. Association analysis can be performed with different tools (e.g., GAPIT [86] and GEMMA [87]) coupled with different model methods [88]).

Based on GWAS, the genetic mechanisms underlying resistance and resilience traits to changing climate have been studied and their causative and predictive factors have been identified in several crops [89–91]. Specific SNPs or InDels have been used for functional marker-assistant selection in breeding programs.

Remarkable works have been conducted in cereals and leguminous to discover SNPs associated with a response to climate change and to develop new resilient crops. In sorghum, GWAS was used to identify SNPs associated with heat stress responses at the vegetative stage under field conditions [92]. SNPs associated with leaf firing and leaf blotching were located in candidate genes (transcription factors, heat-shock proteins, kinases, and phospholipases) that play a role in heat stress response or heat tolerance. A winter barley (*Hordeum vulgare* L.) collection was used to study the effect of CO_2 on biomass traits (aboveground biomass, ears, culms, and leaves) and detect SNPs located in genomic regions involved in the response to CO_2 and crop yield [93]. In chickpea, germplasm collections were used to evaluate drought tolerance, heat tolerance, and yield traits in order to identify significant marker-trait associations to be used for developing superior varieties with enhanced drought and heat tolerance [94]. In addition, Li et al. [95] found SNPs in auxin-related genes associated with yield-related traits under drought conditions.

Genomic selection (GS) may be considered a powerful tool to facilitate the selection of superior genotypes, accelerate the breeding cycle, and reduce the cost of breeding line development [96]. Firstly, a training population (TP) is assembled and is subjected to genotyping and phenotyping for the traits of interest. Then, data are integrated with pedigree information (i.e., a kinship square matrix quantifying pair-wise relationships among population individuals) to build a GS prediction model linking genome-wide marker data to phenotypes. Finally, the model is used on a different set of individuals, which have been previously genotyped but whose phenotype is undetermined (i.e., the breeding population, BP) to get information on their genomic estimated breeding value (GEBV). Clearly, knowing the GEBV of a breeding population allows hinging the selection on marker data without the need of time-consuming and costly phenotyping.

In wheat, GS models were largely developed to identify accessions that best adapt to the negative effects of climate change: FHB resistance [97], heading date as an important component of wheat adaptation [98] and water deficit stress [99]. Recently, Crain et al. [100] disclosed several GS methods in relation to the phenotypic information derived from high-throughput phenotyping platforms. Phenotypic data for drought and heat stresses were analyzed in two environments in more than one thousand advanced wheat lines for grain yield, available at the International Maize and Wheat Improvement Center (CIMMYT).

It was evident that GS, coupled with high-throughput genotyping and phenotyping approaches, increased prediction and selection accuracy in wheat breeding.

3.2.3. Mutation Breeding

Mutation breeding emerged in the middle of the last century with the purpose of artificially developing genetic variability. The use of chemical and physical agents to induce mutations has been successfully adopted worldwide since the 1930s to generate novel alleles, increase genetic diversity, and release mutant varieties in more than 170 different plant species [101]. However, this approach has been almost abandoned due to high costs and controversial opinions of the consequence of mutagenic agents on human health [102].

An alternative to chemical and physical mutagenesis is represented by techniques based on the use of biological agents. Indeed, site-directed mutagenesis and insertional mutagenesis represented alternative forward genetics methods to increase genetic diversity [103]. In the last two decades, mutation breeding has been recovered thanks also to advances in large-scale genome sequencing projects.

Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetic technique based on chemical induced mutagenesis coupled with a sensitive DNA screening-technique [104] which allows the discovery of rare mutations in populations. Traditionally, TILLING protocols were based on the use of enzymatic or physical methods to screen the population and select mutagenized lines. Loss-of-function, gain-of-function, and hypomorphic alleles can be identified and possibly associated with corresponding phenotypes [105].

By combining TILLING with the use of next generation sequencing coupled with multidimensional pooling, Tsai et al. [106] demonstrated that the identification of rare alleles in a population could be effectively expedited. TILLING by sequencing has been successfully applied to discover allelic variants underlying agronomic traits involved in the response to climate change [107,108]. In particular, TILLING was used to discover new allelic variants in the *Hsp26* gene family related to heat stress and thermal tolerance in wheat [109]. Barley mutants were generated by TILLING to study the nucleotide variations in the *era1* (*enhanced response to ABA1*) gene [110], which is differently regulated under drought tolerance in several species including wheat and soybean [111,112].

Modifications to the traditional TILLING or TILLING by sequencing methods have been subsequently proposed. De-TILLING (Deletion TILLING) is an alternative strategy that allows knock-out mutations to be exclusively detected [113]. EcoTILLING is a method developed by Comai et al. [114] to look for natural mutations in individuals. It could be an essential tool for discovering allelic variants responsible for crop adaptation to biotic and abiotic stresses derived by extreme agro-climate conditions [105].

3.2.4. Genome Editing

Genome editing technologies are listed under the larger group of the new plant breeding techniques (NPBT) [115] and can be classified into two categories: oligonucleotide-directed mutagenesis (ODM) and site-directed nucleases (SDNs). Both allow precise directed mutagenesis, gene transfer, and control of gene expression [116].

In the ODM, DNA fragments of 20 to 100 nucleotides in length are chemically synthesized and delivered into plant cells by common methods (e.g., PEG transfection, particle delivery) where they induce mutations in target sites with low efficiency (max. 0.05%) [115].

SDNs are enzymes that can specifically bind to short target DNA sequences ranging from 9 up to 40 nucleotides and exert different biochemical reaction *in situ* (introduction of double-strand breaks (DSBs), methylation, demethylation, acetylation, and deamination) to alter a biological activity (e.g., gene silencing, base editing, gene expression, etc.) [117]. Among all possible biochemical reactions mediated by SDNs, the introduction of DSBs is the most used so far.

In living cells, a DSB can be repaired either by non-homologous end joining (NHEJ) or by homologous recombination (HR); the former seems to be the most frequent in plants. The NHEJ pathway is error-prone, meaning random insertions/deletions (InDels) are usually introduced at the target site (SDN1); this can be exploited to knock-out or knock-down genes (e.g., to study gene function),

alter gene expression, or remove domains (e.g., remove effector binding domain on susceptibility genes [118]). On the contrary, HR is an error-free template-based repair mechanism, which can be used to introduce non-random mutations (SDN2) or insert a large DNA fragment (SDN3) at a target site [115].

SDNs are classified in meganucleases (or homing endonucleases, HE), zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR/Cas). The scientific community and private companies have constantly subjected SDNs to investigation and optimization; however, only with the advent of CRISPR/Cas has genome editing become widely used [119].

Off-target activity is a common issue for all SDNs; however, in plants, the possibility of screening a large edited population and discarding "non-specific editing" makes this issue probably less important compared to the necessary development of transformation protocols, innovation in automation, and tissue-culture-free methods along with investment in transgene-free methods and genomic resources in crops [120]. Indeed, the knowledge on the target and off-target sequences, the availability of an efficient delivery system of SDNs into cells, and the ability to obtain edited homozygous plants are equally important steps in genome editing approaches (SND1-3) that cannot be easily pursued in all crops. For instance, in some important vegetables (pepper, artichoke, and pulses), the development of a reproducible transformation protocol is necessary. As a positive example, in cereals, after years of effort in developing transformation protocols, a recent major breakthrough by Lowe et al. [121] has allowed the boosting of transformation rates in a broad range of accessions.

Transgene-free methods rely on the possibility to transiently express SDNs in plant cells (e.g., protoplasts), and this can be achieved either by the transfer of a DNA-based expression cassette that does not undergo stable integration in the genome [122–124], or alternatively by the transfer of ribonucleoproteins (RNPs) [125,126]. The use of transgene-free methods can lead to genome-edited plants (SDN1 and SDN2 on case-by-case), which are indistinguishable by spontaneously mutated crops or mutants obtained by classical mutagenesis approaches (i.e., ethyl methanesulfonate, ionizing radiation) [115]. Therefore, in the European Union, a distinction in the legislation supporting the approval route toward commercialization of the edited plants deriving by SDN1, SDN2, and SDN3 methods was proposed [127,128]. However, the latest ruling by the European Court of Justice [129] requires that crops generated by using gene-editing techniques such as CRISPR must go through the same lengthy approval process as conventional genetically modified (GM) plants [130]. Surprisingly, no distinctions where made on SDN1, SDN2, or SDN3.

Editing of genes involved in responses to abiotic and biotic stresses has been reported, though only in a limited number of cases and exclusively using SDN1 [116,131]. One of the first successful applications has been the modification by TALEN of the promoter region of the rice bacterial blight susceptibility gene *OsSWEET14.* This change caused the removal of the effector binding element, thus giving resistance to major forms of bacterial blight [132]. Again, by TALEN, it was possible to simultaneously edit three (Mycoplasma Like Organism) homoalleles of the susceptibility gene *MLO*, resulting in powdery mildew resistance in bread wheat [133]. More recently, Nekrasov et al. [134] successfully applied the CRISPR/Cas9 technology in tomato to induce a loss-of-function mutation of the powdery mildew susceptibility gene, *SlMLO1* [135].

Applications to abiotic stresses are still largely confined to model species (e.g., *Arabidopsis*), although some promising results have been recently reported in soybean (drought and salt tolerance by disrupting the *Drb2a* and *Drb2b* genes) [116,136] and more recently announced in cocoa [137]. Abiotic stresses are often controlled by complex genetic mechanisms, which may require simultaneous tuning of different genes (i.e., regulatory sequences, editing of SNPs); on the contrary, for biotic stresses, the knock-out of single genes (i.e., susceptibility genes [138]) is likely to produce the desired phenotype.

New target genes, further technical development allowing both SDN1 and SND2 approaches, and a harmonized legislation on edited crops are necessary to prompt the growth of a novel generation of breeders.

3.3. Bioinformatics and Data Mining: Next Generation Breeding is Going Digital

A large number of crop genomes have been released into the public domain due to major advances in DNA sequencing technologies and bioinformatics. If, on one hand, the availability of a reference genome sequence is of unquestionable value, then on the other hand, it does not represent the diversity within a particular species. As outlined in this work, information on DNA polymorphisms, available through whole genome re-sequencing [139], sequence capture, target-enrichment and re-sequencing methods [140], fractional genome sequencing strategies [141–143], and high-density genotyping arrays [144], is of paramount importance for crop breeding. Indeed, in the last few years, several works addressed the study of genetic diversity in major as well as in "orphan" crops [63,137,145–149].

Aiming to increase the effectiveness of QTL mapping, GWAS, and GS, it is becoming increasingly important to go over the "phenotyping bottleneck" [69] and choose automated technologies for high-throughput plant phenotyping in order to collect measurements of qualitative, agronomical, morphological, and physiological traits. The huge amount of phenotypic data points is challenging in its analysis, management, and accessibility to a greater extent than genotyping data.

As easily understood, bioinformatics is a rapidly expanding field of research as it is essential to extract knowledge from heterogeneous data (i.e., data mining). The analysis of a high number of SNPs and phenotypic data points is demanding and requires an adequate computational infrastructure as well as bioinformatic and shell scripting skills that are beyond the reach of a typical lab. In addition, it is becoming increasingly necessary to integrate various "omics" data (e.g., from genomics and phenomics) with mathematical and statistical models.

There is an urgent need for early training in bioinformatic skills in order to empower plant researchers and breeders to make use of their own data (i.e., for analysis and interpretation) [150]. However, to identify those who are adept at both bioinformatics and plant breeding is difficult and not trivial. A realistic approach is to build interdisciplinary working teams where researchers can share knowledge and expertise to impact on crop improvement.

It seems clear at this point that, similarly to biology, next generation breeding is going digital and that a new figure of breeder is required to cope with recent advances in genomics, transcriptomics, phenomics, and bioinformatics (Figure 2). With this, we do not mean that the next generation breeder will find the field and the computer indistinguishable, but rather that by combining expertise in complementary areas, they will have the greatest potential to be successful in breeding programs in a scenario of increasing climate variability.

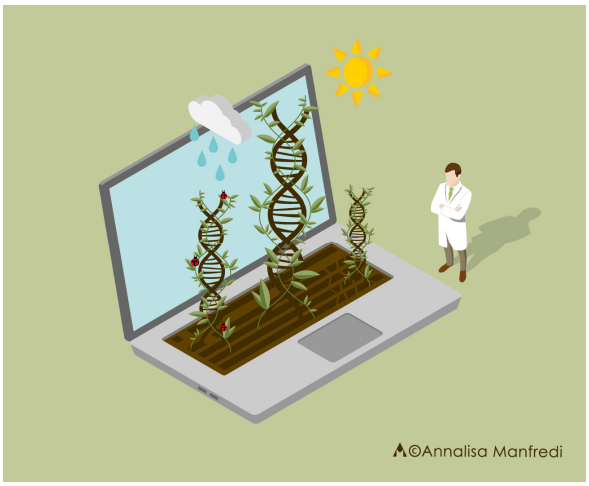

Figure 2. A new figure of breeder is beginning to thrive in the niche created by biotechnological and digital revolution. By combining expertise in complementary areas (open-field trials, wet-lab techniques, big data analysis, and interpretation), they will have the greatest potential to be successful in developing climate-resilient crops.

4. Conclusions

Between 1950 and the late 1960s, the "Green Revolution" dramatically changed the field of agriculture with the aim of providing a solution for the world's food supply problem. Indeed, the global productivity increased drastically, especially in developing countries, thanks to the use of fertilizers, herbicides, pesticides, and high-yield varieties.

In the 21st century, agriculture will face new challenges, largely due to the need to increase global food supply under the declining availability of arable lands and increasing threats from climate change. With respect to this, a white paper was prepared in 2009 by the Food and Agriculture Organization in which the concept of Climate-Smart Agriculture, enabling the ability to cope with food security while facing the challenges of climate change, is emphasized [151]. A prerequisite for climate-smart breeding is the preservation and conservation of genetic resources. Indeed, climate change is altering the behavior of many species, thus affecting ecosystem dynamics. For these reasons, new strategies of germplasm characterization, selection, reproduction, and conservation should be played out so that suitable genetic resources are available to develop cultivars resilient to climate change.

In this review, the most recent popular and breakthrough technologies applied to plant breeding were described and several examples of their applications to breed climate resilient cultivars were provided.

Indeed, breeding for climate-smart agriculture is benefitting from a new revolution, which lays its foundation on the analysis and interpretation of big "omics" data and on NPBT, and which is expected to give fruitful results in the near future.

Nowadays, the breeder's skill set, although it continues to quickly evolve, is rich enough to allow us to start thinking of breeding with different tools than that in the past, as technological improvements in phenotypic and genotypic analysis, as well as the biotechnological and digital revolution, will reduce the breeding cycle in a cost-effective manner [152].

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