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REVIEW ARTICLE

Applying genomic resources to accelerate wheat biofortification

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Abstract

Wheat has low levels of the micronutrients iron and zinc in the grain, which contributes to 2 billion people suffering from micronutrient deficiency globally. While wheat flour is commonly fortified during processing, an attractive and more sustainable solution is biofortification, which could improve micronutrient content in the human diet, without the sustainability issues and costs associated with conventional fortification. Although many studies have used quantitative trait loci mapping and genome-wide association to identify genetic loci to improve micronutrient contents, recent developments in genomics offer an opportunity to accelerate marker discovery and use gene-focussed approaches to engineer improved micronutrient content in wheat. The recent publication of a high-quality wheat genome sequence, alongside gene expression atlases, variation datasets and sequenced mutant populations, provides a foundation to identify genetic loci and genes controlling micronutrient content in wheat. We discuss how novel genomic resources can identify candidate genes for biofortification, integrating knowledge from other cereal crops, and how these genes can be tested using gene editing, transgenic and TILLING approaches. Finally, we highlight key challenges remaining to develop wheat cultivars with high levels of iron and zinc.

Introduction

Wheat provides a fifth of the overall calories consumed by humankind (FAO 2017), and is a staple food crop for about 30% of the population, especially in developing countries (Lobell et al. 2011). However, low levels of micronutrients, such as iron and zinc in wheat grain, can lead to micronutrient deficiencies in people whose diets consist mainly of cereals such as wheat. Globally, micronutrient deficiency affects 2 billion people, with inadequate levels of iron and zinc consumption common (Beal et al. 2017). The WHO estimates that globally one in three women of reproductive age are anaemic, most commonly caused by iron deficiency (WHO 2018), and iron deficiency caused the loss of over 46,000 disability-adjusted life years during 2010 alone (Murray and Lopez 2013). Approximately 17% of the world's population is also facing inadequate zinc intake (Wessells and Brown 2012), which causes stunted growth and increases the risk of child mortality. These micronutrient deficiencies are most commonly found in the Middle East, South Asia and North Africa (Beal et al. 2017).

The micronutrients in diets can be increased through food supplementation, industrial fortification or biofortification. Biofortification is a strategy to increase the level of minerals and vitamins in crops by applying genomic, biotechnology and breeding techniques (Bouis and Saltzman 2017; Garg et al. 2018). While dietary requirements are provided by varied diets and/or food supplements in developed countries, people living in developing countries may have a more limited diet and no access to dietary supplements (Ward 2014). Biofortification is a sustainable and long-term approach to overcome micronutrient deficiency compared with fortification or dietary supplements that require ongoing investment (de Valença et al. 2017). Since wheat is eaten by 2.5 billion people globally (CIMMYT 2017), increasing the iron and zinc content of wheat grain could have a significant impact on human health by reducing iron and zinc deficiencies.

Due to the importance of this topic, there are many recent review articles (e.g. Cakmak and Kutman 2018; Connorton and Balk 2019; Ludwig and Slamet-Loedin 2019; Saini et al. 2020), which summarise agronomic and genetic

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approaches to wheat biofortification. However, limited attention has been paid to how the recent developments in wheat genomics can be applied specifically to biofortification. Therefore, here we first describe the genomic resources made available for wheat in the past few years, which include fully annotated genome sequences, gene expression atlases, gene networks and sequenced mutant populations, and explore how these genomic resources can be applied to biofortification. Secondly, we discuss existing approaches to biofortification (e.g. Quantitative trait loci (QTL) and Genome-wide association studies (GWAS) studies), and explore how genomic resources can contribute to these approaches, for example, by accelerating genetic marker development. Finally, we consider how the availability of genome sequences and gene models now facilitates gene-centric approaches to biofortification through transgenics, gene editing or the use of sequenced mutants.

New genomic resources for biofortification

Although biofortification has been considered for many years, it has proved challenging to biofortify wheat through conventional breeding programmes, in part due to high costs of measuring micronutrient content that is required for a traditional phenotype-driven approach. Marker-assisted selection could provide a lower-cost route to increase micronutrient content. However, this approach has been impeded by the lack of a genome sequence for wheat, which is difficult to assemble due to the size of the wheat genome (16 Gb), its high repetitive content (>85%) and polyploid nature. Without a genome sequence, it has been difficult to design genetic markers, map loci regulating iron and zinc content or use gene-targeted approaches based on knowledge from model species. Rapid developments in genomic resources for wheat now present an opportunity to overcome many of these difficulties, and accelerate the development of biofortified wheat cultivars (Fig. 1).

Identification of candidate genes for biofortification using novel genomic resources

Several genome assemblies have been constructed for the reference wheat landrace Chinese Spring (Clavijo et al. 2017; IWGSC 2014; IWGSC et al. 2018; Zimin et al. 2017) with various levels of completeness and annotations (reviewed in Borrill et al. (2019)). In 2018, an annotated chromosome-level genome sequence (RefSeqv1.0) was published, which has a total assembly size of 14.5 Gb, representing 94% of the whole genome (IWGSC et al. 2018). The high contiguity of this genome assembly will facilitate genetic mapping of loci involved in iron and zinc accumulation. In total 107,891 high-confidence gene models were annotated, which will enable genome-wide analysis of biofortification-related gene families, and aid the interpretation of loci identified in QTL and GWAS studies. For example, we have identified the members of the wheat NRAMP family by orthology with rice (Table 1). This family illustrates that analysing gene families with up-to-date reference sequences will be necessary, for example, several of the NRAMP gene models were split across contigs or missing in previous assemblies, but are now complete (see Borrill et al. (2014) for details of 2014 Chinese Spring Survey assembly). It is important to note that despite significant improvements, the RefSeqv1.1 gene annotations are not perfect, for example, two out of the 24 NRAMP genes are incomplete (Table 1). These known limitations are being addressed by ongoing work to improve the Chinese Spring genome assembly and its annotations. Furthermore, high-quality genome sequences and annotations are in progress for additional cultivars (www.10wheatgenomes.com), which will allow comparison and independent validation of gene models. Genome-wide analysis has been carried out for several gene families involved in iron and zinc transport, including the MFS–zinc-induced facilitator-1 like (Sharma et al. 2019a), metal tolerance proteins (Vatansever et al. 2017), yellow stripe-like transporter (Kumar et al. 2019) and the vacuolar-iron transporter-like (Sharma et al. 2019b), identifying potential candidate genes for biofortification. However, several of these studies use the earlier TGAC genome assembly (Clavijo et al. 2017) rather than the latest RefSeqv1.0 reference sequence (IWGSC et al. 2018), which may mean that some gene models are missing or truncated, as we found for the NRAMPs (Table 1).

Many of the gene families involved in iron and zinc transport are large multigene families; therefore, to use a gene-centric approach to biofortification, it will be necessary to identify the appropriate family members for further investigation. Phylogenetic approaches can reveal which family members are most closely related to genes characterised in other species. This approach was used to identify *TaZIP* genes (Evens et al. 2017) and their roles in transporting zinc were confirmed using heterologous expression in yeast. Further information about family members can be gathered by exploring gene expression patterns. The expVIP gene expression atlas ((Borrill et al. 2016; Ramirez-Gonzalez et al. 2018); www.wheat-expression.com) provides access to over 1000 RNA-seq samples to explore gene expression patterns across a wide range of tissues, developmental stages and stress conditions. For example, analysis of the NRAMP family with this resource reveals that *TaNRAMP3* is expressed more highly in the grain, whereas *TaNRAMP5* is expressed more highly in the root and spike (Table 1). Rapid analysis can also be

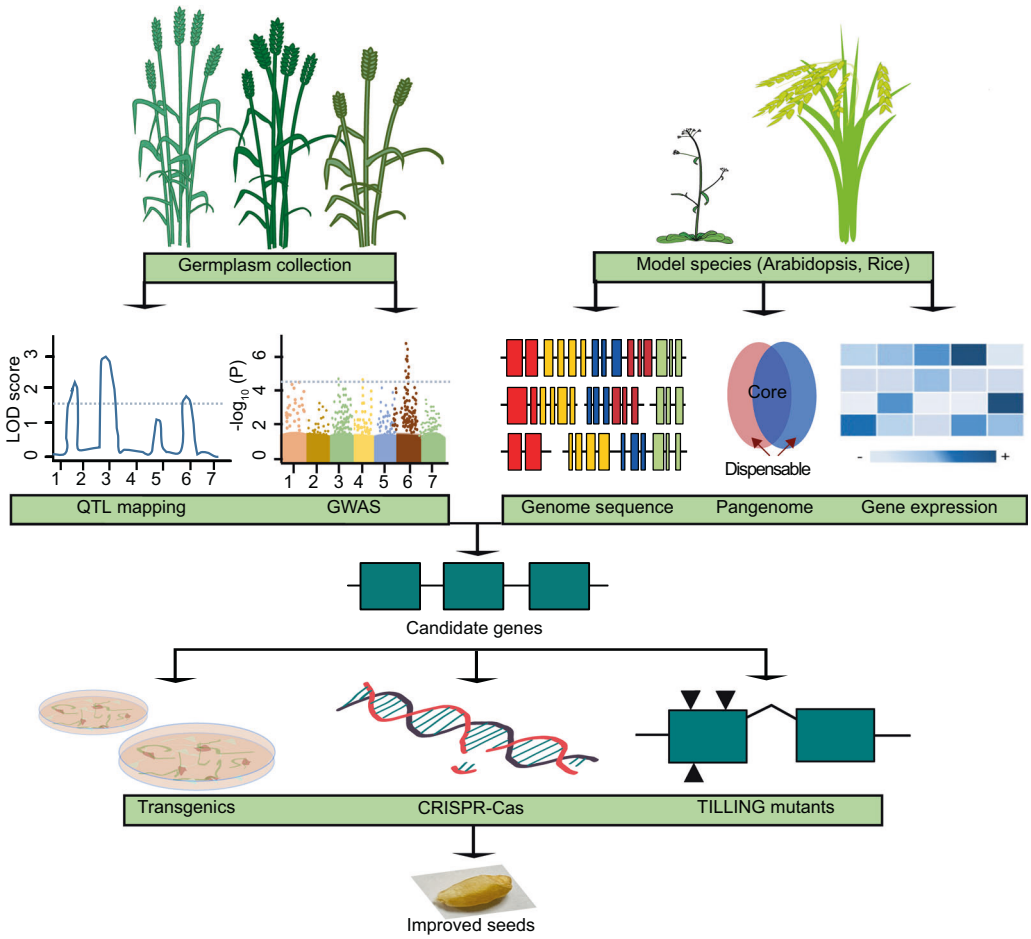
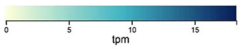


Fig. 1 Schematic representation of Fe/Zn biofortification using modern genomic resources. Candidate genes for biofortification can be identified through two routes. Germplasm collections can be screened for lines with high Fe/Zn content. New genome sequences will facilitate the identification of genetic loci controlling Fe/Zn content by GWAS or QTL mapping, and candidate genes can be identified within these loci using the gene annotations. A second approach uses information about gene function from model species to identify orthologous candidate genes in the wheat genome. Gene expression and sequence or the presence/absence variation from pangenomes can help refine candidate genes. Candidate genes identified by either route can be validated using transgenic, CRISPR–Cas or TILLING approaches, before being introduced into breeding programmes. The *Arabidopsis* diagram (Bouché 2018) is used under a CC-BY 4.0 license.

carried out using the pictographic eFP browser ((Ramirez-Gonzalez et al. 2018; Winter et al. 2007); https://bar.utoronto.ca/efp_wheat/cgi-bin/efpWeb.cgi), which displays expression patterns across 70 different tissues and developmental stages. It is worth noting that these resources only determine the expression of genes annotated in the Chinese Spring reference sequence, and custom mapping will be necessary to understand the expression patterns of genes only present in other cultivars. An additional limitation of these resources is the lack of RNA-seq data from iron- and zinc-associated studies, but the increasing use of RNA-seq as an experimental technique will likely address this gap, with one study on iron-starvation responses in wheat seedlings recently published (Kaur et al. 2019).

Beyond understanding gene expression levels at a single-gene level, it is important to consider how genes act together to regulate phenotypes. Many different genes are expected to be involved in iron and zinc uptake, transport and accumulation. Gene networks could be used to identify interaction and coordination between these genes. The use of networks to understand biology has been relatively limited in wheat, although studies have used co-expression networks to identify genes and pathways associated with diverse traits, including Fusarium head blight (Kugler et al. 2013), meiosis (Alabdullah et al. 2019) and spike architecture (Wang et al. 2017). Furthermore, a set of co-expression networks have been developed for specific tissues (root, leaf, grain and spike) and abiotic or disease stress conditions (Ramirez-Gonzalez et al. 2018). An integrated network has been developed for 850 RNA-seq samples, which enables co-expression to be studied across a range of tissues (IWGSC et al. 2018), and these data have been

Table 1 Gene identification and expression patterns of wheat NRAMPs. Wheat NRAMPs were identified from their rice orthologues, using Ensembl Plants phylogenetic trees (Howe et al. 2020). Gene expression (transcripts per million, tpm) data are shown for the Chinese Spring development study in expVIP (Borrill et al. 2016; Ramirez-Gonzalez et al. 2018). Expression values shown are for the RefSeqv1.1 genes, with expression levels summarised per tissue.



Rice NRAMP (RAP locus ID)	Wheat ortholog	Genome	TGAC gene ID	RefSeqv1.1 gene ID[a]	R	L	G	S
OsNRAMP1 (Os07g0258400)	*TaNRAMP1*	A	*TRIAE_CS42_7AL_TGACv1_556173_AA1757430*	*TraesCS7A02G327300*				
		B	*TRIAE_CS42_7BL_TGACv1_577015_AA1862810*	*TraesCS7B02G227900*				
		D	*TRIAE_CS42_7DL_TGACv1_605996_AA2008810*	*TraesCS7D02G324000*				
OsNRAMP2 (Os03g0208500)	*TaNRAMP2*	A	*TRIAE_CS42_4AS_TGACv1_307585_AA1021690*	*TraesCS4A02G050500*				
		B	*TRIAE_CS42_4BL_TGACv1_320879_AA1050740*	*TraesCS4B02G254300*				
		D	*TRIAE_CS42_4DL_TGACv1_344138_AA1144210*	*TraesCS4D02G254100*				
OsNRAMP3 (Os06g0676000)	*TaNRAMP3*	A	*TRIAE_CS42_7AL_TGACv1_557364_AA1780410*[b]	*TraesCS7A02G464300*				
		B	*TRIAE_CS42_7BL_TGACv1_576780_AA1854780*	*TraesCS7B02G364800*				
		D	*TRIAE_CS42_7DL_TGACv1_603581_AA1985930*[b]	*TraesCS7D02G451900*				
OsNRAMP4 (Os02g0131800)	*TaNRAMP4*	A	*TRIAE_CS42_6AS_TGACv1_487188_AA1568800*	*TraesCS6A02G108500LC/ TraesCS6A02G108600LC*[b]				
		B	*TRIAE_CS42_6BS_TGACv1_514283_AA1657800*	*TraesCS6B02G168400LC*				
		D	*TRIAE_CS42_5BL_TGACv1_404828_AA1312170*	*TraesCSU02G077000/ TraesCSU02G124800LC*[b]				
OsNRAMP5 (Os07g0257200)	*TaNRAMP5*	A	*TRIAE_CS42_4AS_TGACv1_306761_AA1013050*	*TraesCS4A02G004400*				
		B	*TRIAE_CS42_4BL_TGACv1_322206_AA1069640*	*TraesCS4B02G300600*				
		D	Absent	*TraesCS4D02G299400*				
OsNRAMP6 (Os01g0503400)	*TaNRAMP6*	A	*TRIAE_CS42_3AS_TGACv1_211973_AA0696140*[b]	*TraesCS3A02G195100*				
		B	*TRIAE_CS42_3B_TGACv1_222100_AA0757350*[b]	*TraesCS3B02G230400*				
		D	Across multiple contigs	*TraesCS3D02G206000*				
OsNRAMP7 (Os12g0581600)	*TaNRAMP7*	A	*TRIAE_CS42_5AS_TGACv1_394226_AA1279000*	*TraesCS5A02G072200*				
		B	*TRIAE_CS42_5BS_TGACv1_423603_AA1380480*	*TraesCS5B02G078700*				
		D	*TRIAE_CS42_5DS_TGACv1_456926_AA1479950*[b]	*TraesCS5D02G084900*				
–	*TaNRAMP8*	A	*TRIAE_CS42_4AL_TGACv1_290083_AA0981450*	*TraesCS4A02G237200*				
		B	*TRIAE_CS42_4BS_TGACv1_328307_AA1085990*	*TraesCS4B02G078000*				
		D	*TRIAE_CS42_4DS_TGACv1_361580_AA1170390*	*TraesCS4D02G076500*				

R roots, *L* leaves/shoots, *G* grain, *S* spike.

[a]Gene IDs are shown for the v1.1. gene annotation on the RefSeqv1.0 genome sequence.

[b]Partial sequence.

incorporated in Knetminer (Hassani-Pak et al. 2016) (https://knetminer.rothamsted.ac.uk/KnetMiner/) for easy accessibility. Knetminer also provides access to a network of predicted transcription factor targets (IWGSC et al. 2018), with the predictions validated using an independent RNA-seq dataset (Harrington et al. 2019).

To illustrate the potential of these networks to understand the regulation of iron and zinc genes, we searched Knetminer using the three homoeologs of *NRAMP5*, which are highly expressed in the roots (Table 1). The network predicted that a NAC transcription factor (*TraesCS6B02G416400*) regulates all three homoeologs of *NRAMP5*, and three homoeologs of a YABBY transcription factor (*TraesCS4A02G058800*, *TraesCS4B02G245900* and *TraesCS4D02G245300*) were predicted to regulate the A and B homoeologs of *NRAMP5*. These predicted transcription factors could serve as a starting point to explore the network of genes transcriptionally regulating *NRAMP5;* see Adamski et al. (2020) for a further case study of using gene networks to identify candidate genes in wheat. Together, these network-based resources provide a powerful entry point to identify genes regulating iron and zinc in wheat, including novel genes not identified in other plant species.

Moving beyond a single reference sequence

The identification of genes within the Chinese Spring reference landrace represents a major step forward; however, based on genome-scale analysis in other grass species, including *Brachypodium distachyon* (Gordon et al. 2017) and rice (Zhao et al. 2018), we expect that there will be a large variation in gene content between different wheat cultivars. Within hexaploid wheat, current efforts are generating high-quality sequences for additional cultivars (www.10wheatgenomes.com), which may reveal differences in the content of iron- and zinc- associated genes. Studies have used exome capture to sequence exonic regions across hundreds of wheat lines (He et al. 2019; Pont et al. 2019), which could be leveraged to identify novel variation within proposed biofortification genes.

The highest concentrations of iron and zinc are often observed in wild progenitor species (Velu et al. 2019),

which may contain genes not found in hexaploid wheat. Therefore, it may be informative to compare the iron- and zinc-associated gene families in genome sequences of elite wheat cultivars with the genome sequences available for diploid and tetraploid progenitors of wheat (Avni et al. 2017; Ling et al. 2018; Luo et al. 2017). Efforts are also being made to sequence multiple accessions of wild cultivars (e.g. Open Wild Wheat http://www.openwildwheat.org/), which could reveal additional variation in gene content. In silico identification of cultivars with promising gene content (based on gene family information) could be cross-referenced with efforts to catalogue the variation in Fe and Zn content within wild accessions (reviewed in Velu et al. (2014)).

Discovery of molecular markers associated with iron and zinc content

Identifying and targeting gene families already known in other plant species to be involved in iron and zinc transport may be an effective approach for biofortification; however, it is possible that this approach will not identify all the genetic regulators of iron and zinc content in wheat because even in model plant species, the biological pathways are not fully understood. Genetic mapping approaches can identify novel loci and genes affecting iron and zinc content. The genome sequences now available will accelerate the mapping of loci for iron and zinc content, and facilitate the development of molecular markers to use these loci in breeding programmes.

Quantitative trait loci (QTL) mapping

In 1997, the first QTL for iron and zinc content in wheat was mapped, on chromosome 6BS, in a recombinant inbred line population generated from a cross between durum wheat and wild emmer (*Triticum turgidum*) (Joppa et al. 1997). This QTL, *Gpc-B1*, conferred an 18% increase in iron and a 12% increase in zinc content, and the gene underlying the QTL was identified to be the NAC transcription factor *NAM-B1* (Distelfeld et al. 2007; Uauy et al. 2006). Many QTL studies have been conducted to explore the genetic regions for iron and zinc contents in wheat, which have been recently reviewed elsewhere (Garcia-Oliveira et al. 2018; Saini et al. 2020). The QTLs identified within each study were often different from each other due to differences in populations, environments and marker sets, which greatly affect the location and precision of the QTL identified. Using the new reference sequence as a standard, it may now be easier to compare the physical position of the QTL identified in different studies, by mapping the markers onto the genome sequence. This may enable the identification of consistently performing QTL for enhanced iron and zinc content. It may also be possible to identify multiple small-effect QTL, which previously could not be detected due to limited marker density. However, deploying many QTL simultaneously through marker-assisted selection may be difficult in large breeding populations due to practical limits on the number of markers that can be used. Instead, small-effect QTL within the same genetic region could be stacked using marker-assisted selection to form a haplotype containing multiple beneficial QTL. This haplotype could then be selected as a single unit by marker-assisted selection, reducing the number of markers required at later selection steps. This approach could also allow QTL for increased iron and zinc content to be combined with QTL for other beneficial traits, e.g. yield or disease resistance, which are more likely to already be under marker-assisted selection, thereby providing added value to marker usage.

Increasing grain iron and zinc simultaneously may also be facilitated by their significant positive correlation in many environments, which may be caused by the colocalisation of QTL for iron and zinc that are underpinned by separate genes or by pleiotropic effects of a single gene. For example, the transcription factor *NAM-B1* affects both iron and zinc content by altering the rate of senescence, which influences remobilisation of multiple micronutrients (Uauy et al. 2006), illustrating pleiotropy. However, in most cases, the gene(s) underlying QTL affecting iron and zinc have not been identified (e.g. Crespo-Herrera et al. 2016; Krishnappa et al. 2017; Tiwari et al. 2016), so the molecular mechanisms explaining the colocalisation of QTL for iron and zinc are unknown. Nevertheless, for breeding biofortified wheat, the colocalisation of QTL offers an opportunity to improve iron and zinc content in wheat grains simultaneously.

Genome-wide association studies (GWAS)

GWAS can be used to dissect the genetics underpinning traits using a diverse population, rather than a biparental population that is required for QTL mapping. GWAS can therefore include a broader genetic base and can be less time consuming than QTL mapping because the development of segregating populations is not required. Furthermore, GWAS benefits from increased historic recombination that can lead to higher-resolution mapping than QTL mapping, although it is necessary to control for population structure (Mitchell-Olds 2010).

GWAS has been used in a few studies to dissect the genomic regions associated with iron and zinc contents in wheat, although it has been used less than QTL mapping. A study of 369 elite European wheat cultivars identified 41 marker-trait associations for iron content, many of which were located on chromosome 3B (Alomari et al. 2018), and

a smaller panel of 123 synthetic hexaploid wheats was used to identified three marker-trait associations for iron and thirteen for zinc (Bhatta et al. 2018). The detection of marker-trait associations by GWAS is highly dependent on the genetic and phenotypic variation within the panel. Therefore, the characterisation of iron and zinc contents in genetically diverse panels, such as the HarvestPlus Association Mapping panel (Velu et al. 2016) and germplasm derived from the Watkins landraces (Khokhar et al. 2020), will be valuable for GWAS studies. Indeed, Velu et al. (2018) discovered 39 marker-trait associations for zinc content, including major loci on chromosomes 2 and 7 using the HarvestPlus Association Mapping panel. Although GWAS can provide a higher resolution than QTL mapping studies, it is still difficult to identify the causal single-nucleotide polymorphism in wheat because the region identified often contains many genes. However, the annotated genome sequence now allows the identification of putative candidate genes, for example, NAC transcription factors and transmembrane proteins were identified by Alomari et al. (2018). However, further work will be required to confirm whether these candidate genes underpin the variation observed in iron content.

GWAS and QTL studies to date have revealed that iron and zinc content are controlled by a large number of loci that mainly have small effects. Furthermore, different loci have been identified in different populations. For these reasons, it has been challenging to move from discovery of loci to implementation in breeding programmes. This challenge has been compounded by the lack of an accurate genome sequence, which has impeded the development of genetic markers. The lack of a complete genome sequence also affects the precision with which loci can be identified in GWAS and QTL studies. However, the RefSeqv1.0 assembly has enabled the identification of candidate genes underpinning QTL for other traits, such as stem solidness, which could not be identified using older less-contiguous assemblies (IWGSC et al. 2018), suggesting that more precision will be possible for iron and zinc QTL. Moving forward, the high-quality genome sequence will enable the accurate identification of regions affecting iron and zinc content, and accelerate the development of markers for use in breeding programmes. Extensive genotyping has already been carried out on panels of wheat cultivars, landraces and wild relatives (reviewed in Borrill et al. (2019)), and therefore it will be possible to test in silico for variation in markers identified (e.g. using Cereals DB data available at Ensembl Plants (Howe et al. 2020)). This will give an indication of variation in these markers, and help to predict how effective they will be for selection in breeding programmes. Furthermore, it may be possible to identify wheat lines in which several beneficial alleles for iron and/or zinc content are located with a haplotype block. Markers to select for the entire haplotype block could be used in breeding programmes, which would require fewer markers than selecting for the individual beneficial loci. This haplotype-based approach could be more cost-effective and practical within breeding programmes, as well as being more effective at enhancing iron and zinc content than deploying individual QTL, assuming additivity of individual QTL within the haplotype block.

Transgenic approaches

QTL and GWAS are natural diversity-based approaches, which may take a long time to implement significant increases in micronutrient content. In contrast, transgenic approaches represent a rapid route to increase micronutrient contents with the possibility of larger gains in micronutrient content. In addition, transgenic approaches may not only increase iron and zinc level in the whole grains, but also enhance the accumulation of iron and zinc in the endosperm. Since the outer tissues of wheat grain are largely removed by milling, increasing micronutrient content in the endosperm could be more beneficial to increase human consumption of iron and zinc. Fewer studies have been carried out in wheat than in the model cereal rice; however, increased transformation efficiencies (Hayta et al. 2019) and novel strategies to transform elite lines (reviewed in Borrill (2020)) will facilitate rapid characterisation of gene function in wheat directly. Despite technological improvements, regulatory hurdles will likely delay the adoption of transgenic wheat lines in agriculture. Nevertheless, transgenic studies will help to elucidate the function of candidate genes, whether they are identified through mapping approaches or by orthology to genes identified initially in other plant species, as described below.

The first transgenic approach to increase iron content in wheat aimed to increase storage of iron in the endosperm by expressing the ferritin genes (*TaFer1*/*TaFer2*), which encode iron-binding proteins, under an endosperm-specific promoter. In wheat, as in rice, this resulted in an increased level of iron in the grain, which was 50–85% higher than in wild-type plants (Borg et al. 2012). A similar rationale (increasing iron storage in the endosperm) underpinned a more recent study in which *TaVIT2*, a vacuolar-iron transporter, was expressed under an endosperm-specific promoter, which resulted in a twofold increase in iron in wheat flour compared with control lines (Connorton et al. 2017). Other studies have manipulated the uptake and translocation of iron and zinc by overexpressing *OsNAS2*, which encodes a nicotianamine synthase enzyme. Nicotianamine plays a key role chelating and transporting iron and zinc in higher plants, and hence can affect translocation to the grain. Nicotianamine is also a precursor for 2′-deoxymugenic acid,

a root-secreted phytosiderophore that chelates Fe^{3+} for uptake by the roots. Two studies showed that over-expression of *OsNAS2* under the maize ubiquitin promoter increased wheat grain iron content by up to 2.1- (Singh et al. 2017) and 1.4-fold (Beasley et al. 2019). Increases in zinc content were also observed in these lines of up to 3.7-fold (Singh et al. 2017). The effects on iron and zinc content were also retained under field conditions without adverse effects on performance (Beasley et al. 2019), illustrating the agronomic potential of this approach. Transgenic studies have been impeded by a lack of genomic information for wheat, but now that high-quality gene sequences are available, and transformation efficiencies are increasing, it is likely that transgenic studies will play a significant role in identifying functions of genes in iron and zinc transport in wheat.

Alternatives to traditional transgenics

Whilst transgenic approaches could be effective at increasing iron and zinc content in wheat, to date, there have been no genetically modified (GM) wheat cultivars released. Furthermore, GM cultivars may be delayed by regulatory hurdles. This has been the case for golden rice (Dubock 2017), which was developed in 2000, yet its first approval to combat vitamin A deficiency in a developing country only came at the end of 2019 in the Philippines (International Rice Research Institute 2019). Gene editing, such as CRISPR–Cas9, may escape some of these hurdles, because many countries will not regulate these technologies as GM, although they are considered GM in Europe. Alternatively, mutagenesis can produce alterations to gene function, which would not be considered GM, and therefore can be readily applied in breeding programmes.

CRISPR–Cas

Gene-editing technologies, such as CRISPR–Cas, allow precise manipulation of gene function by the induction of small deletions that lead to frameshifts and knockouts (Zhang et al. 2016). In wheat, simultaneous editing of the three homoeologs has been demonstrated (Cui et al. 2019; Zhang et al. 2016), although only a low percentage of transgenic lines have all three homoeologs edited. More complex editing, such as targeted gene insertion, single-base editing and modifications to epigenetic marks, is also now possible (for review see Zhang et al. (2019)). The use of CRISPR–Cas may enable the rapid modification of genes for biofortification directly in elite wheat cultivars, avoiding many generations of backcrossing to remove deleterious alleles introduced by conventional crossing programmes. Gene editing can also be used to increase phenotypic variation. For example, in tomato, an expanded range of fruit sizes was generated by targeting promoter regions of *CLAVATA3* (Rodríguez-Leal et al. 2017), and similar approaches could be used to increase variation in wheat iron and zinc content by targeting regulatory genes. However, for this approach to be effective, a better understanding of the genes regulating iron and zinc content in wheat grains would be required.

Mutagenesis

The use of mutagenised populations, which are not subject to GM regulations, is an alternative route to manipulate gene function, but until recently, finding mutations in a gene of interest using conventional TILLING (Targeting Induced Local Lesions IN Genomes) approaches was extremely laborious. Sequenced mutant populations in hexaploid and tetraploid wheat (Krasileva et al. 2017) have now been developed, which enable the rapid identification of mutations in genes of interest in silico. Although the mutations were originally identified using the 2014 IWGSC reference genome, they have been reanalysed using the RefSeqv1.0 genome, and are available on the Ensembl Plants website (Howe et al. 2020). Over 10 million mutations are present in the two sequenced populations, with 60% of genes having a premature termination codon or splice junction variation expected to lead to a truncation (Borrill et al. 2019). For genes where a truncation mutation is not available, missense mutations are likely to be available, although predicting the phenotypic effects of missense alleles can be more difficult, and testing several lines may be necessary.

Crossing mutant lines in different homoeologs together will likely be required for many genes to generate phenotypic effects, due to redundancy between homoeologs (for review see Borrill et al. (2015)). This approach has been applied to increase provitamin A content in durum wheat (Sestili et al. 2019), and could readily be applied to iron and zinc, if suitable target genes are identified. To use the TILLING lines in breeding, it will be necessary to carry out backcrossing to remove background mutations, and to move the mutations of interest (frequently mutations in all three homoeologs) into an elite cultivar adapted to the target region. This process of marker-assisted backcrossing will take a significant amount of time, although speed breeding can be used to accelerate generation times (Watson et al. 2018). Another use of mutagenised populations could be to screen for increased iron and zinc content, which may be beyond the range observed in natural populations, as has been shown for gamma-irradiated wheat lines (Kenzhebayeva et al. 2019). These mutagenesis-based approaches could be used to develop wheat cultivars with enhanced iron and zinc content, without being subject to GM regulations, which could delay their use in the field.

Conclusions and future perspectives

The availability of high-quality genomic resources for wheat will underpin efforts to increase iron and zinc content in wheat grains. These resources will help to map genetic loci for iron and zinc content, and allow knowledge from model species to be applied in wheat through transgenic, CRISPR–Cas or TILLING approaches.

Despite these improved resources, several challenges remain for biofortification in wheat. Firstly, to gain the maximum information from existing studies, it would be useful to unify studies to a common reference sequence, and to standardise nomenclature. This will take a concerted effort from the global community, and this challenge will be magnified by the release of reference sequences for additional cultivars. However, despite the challenges associated with data integration, the availability of multiple reference sequences will provide great benefits, for example, accelerating gene cloning by ensuring that the reference sequence used for physical mapping contains the gene of interest. Another major challenge is to move from being able to increase iron and zinc content in wheat grain in individual wheat cultivars, to introducing these benefits into breeding programmes, and ensuring that the improved cultivars reach the people who need them the most. Several international organisations, including CIMMYT and HarvestPlus, are already working towards this goal, and the new genomic resources will help to accelerate progress. The foundations have now been laid to increase iron and zinc content in wheat, which could have significant effects on global human health. To ensure that this goal is achieved, an integrated approach that goes beyond technology alone will be required. Working with consumers, public health officials and governments will be essential for the new wheat cultivars to achieve their potential to alleviate micronutrient deficiency.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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