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Genetic engineering of wheat – current challenges and opportunities

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Wheat is one of the major staple food crops grown worldwide; however, productivity in cereal crops has not kept pace with the world population growth. A significant increase in wheat production (>40% by 2020) is needed simply to keep up with the growing demand. This increase is unlikely to be achieved by conventional plant breeding methods because of the limited gene pool available. The application of recombinant techniques to improve wheat quality and yield is not only desirable but also has potential to open up new opportunities. Although there has been significant progress in developing gene-transformation technologies for improving these traits, this remains an important challenge for plant biotechnology. Obstacles to translate the full potential of the genomic era to wheat breeding include the need to develop elite wheat varieties without selectable markers, introducing minimal or nil intergenic DNA and social and market issues concerning genetically engineered food products.

Introduction

Wheat is the major crop in basic food commodities, followed by coarse grains and rice. According to the Food and Agriculture Organization of the United Nations (FAO; http://www.fao.org/), the worldwide production of wheat was >500 million tonnes in 2004 (*FAO Food Outlook Report,* April 2005; http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/007/J5051e/J5051e00.htm). Moreover, it is a staple food, of which the average per-capita global consumption in 2005 was 68 kg: 61 kg and 95 kg in developing and developed countries, respectively. Wheat is also used as animal feed, with more than 110 million tonnes being used for this purpose in 2004. According to a recent FAO report (*FAO Food Outlook Report*, September 2005; http://www.fao.org/documents/show_cdr.asp?url_file=/docrep/008/J6217e/J6217e00.htm), global cereal production will be inadequate to cover the expected consumption in the marketing year 2005–2006, pointing to a larger than anticipated drawdown in global cereal stocks. In addition, cereal inventories held by the major exporting countries have been forecasted to decline.

The world population is currently increasing by around 100 million per year and is expected to exceed 10 billion by 2050, with a concomitant requirement to double the food produced from the same amount of arable land. In addition, an upward trend (2.4-fold increase) in per-capita income, with resulting changes in dietary requirements, has been forecasted [1]. To ensure global, political and social stability, increasing sustainable food production, equitably, without compromising environmental integrity remains a major challenge. Genetic engineering offers the opportunity to increase the efficiency of food production by avoiding losses due to disease and pests, by increasing tolerance under adverse conditions and breeding crops with novel desirable characteristics, such as reduced allergenicity [2–4], improved nutritional qualities [5–7], hybrid seed production [8–10] and improved plant productivity [11,12]. These enhancements cannot be achieved by conventional breeding methods alone.

Plant biotechnology has made significant advancements during the past decade, and several crops are now grown commercially. Globally, the area covered by genetically modified (GM) crops increased from 1.7 million hectares to 90 million hectares between 1996 and 2005, with an increasing proportion grown in developing countries. The dominant GM crops are currently soybean, corn, cotton and canola. Furthermore, engineered traits, such as herbicide tolerance and insect resistance, have resulted in a significant reduction in herbicide and insecticide use. The increased hectarage and the adoption of GM crops by five major developing countries (China, India, Argentina, Brazil and South Africa) have implications for the future use and acceptance of GM crops worldwide. In addition, GM crops are also being explored as a pharmaceutical production platform [13].

Wheat genome characteristics

Wheat shares a remarkably parallel evolutionary history with humans [14]. It is also adapted to temperate climates, whereas other major cereals (e.g. corn and rice) are suited to tropical conditions. To meet human needs by 2050, grain production must increase at an annual rate of 2% [14]. Moreover, wheat has the largest genome of the three major agricultural cereal crops – 16 000 Mb – which is approximately eightfold and 40-fold larger than those of corn and rice, respectively [15]. Table 1 shows a comparison of key genome features between wheat and rice, and Figure 1 depicts the evolutionary history of major cereal crops. Bread (or hexaploid) wheat, *Triticum aestivum* L., is composed of three genomes: A, B and D.

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Table 1. Comparison of key genome features of rice and wheat

Genome characteristics	Wheat	Rice
Size	~16 000 Mb	430 Mb
Polyploidy	Yes	No
Chromosome number	Three genomes (A, B and D); 2n=42; function like diploid	2n=12
Level of transposable elements (TE)	70% of genome comprises TE	22% of genome comprises TE
Repeated sequences	90% of genome comprises repeated sequences	42% genome comprises repeated sequences
Gene clusters	Gene clusters on chromosomes are separated by long stretches of TE	Homogenous distribution
Distribution of agronomically important genes on the chromosome	Chromosome-specific and not necessarily triplicated in the hexaploid genome (e.g. *rust* and *powdery mildew*, *pairing control* gene, *grain hardness* gene)	Not an issue for concern with diploid genome of rice

These exhibit little colinearity due to their rapid evolution [16] – many agronomically important genes are not triplicated [14]. Progress in genomic and genetic engineering research in wheat has lagged behind that in the other major crops because of its large genome. However, the economic and social imperative has driven genomic research in crops towards the identification of 500 000 expressed sequence tags (ESTs) from wheat, which is the largest available for all plant species [17].

Challenges faced by wheat genetic engineering

Gene-transformation technology complements the contemporary focus of genomics and is crucial to gene discovery and function, and variety improvement. It is defined as an enabling technology for the introduction of novel genes into commercial lines from unrelated sources, a feat that is impossible by conventional breeding. Thus, given the significant challenges in developing the associated gene-transformation technology, it is not surprising that wheat does not form part of the current GM crop portfolio.

Wheat has been successfully transformed using microprojectile bombardment and *Agrobacterium*-mediated transformation methods. Both of these methods involve delivery of the transgene to callus tissues, followed by selection of transformed cells and regeneration of plantlets carrying the gene of interest. The first successful wheat transformation was achieved using the microprojectile bombardment method [18]: this involves bombarding cells with DNA-coated gold or tungsten particles. The *Agrobacterium*-mediated transformation method exploits the unique ability of this bacterium to introduce the transgene into plant cells. Both methods have their merits and limitations: microprojectile bombardment is considered versatile and easy to adapt in terms of gene delivery, whereas the *Agrobacterium*-mediated method represents a simple, low-cost alternative to microprojectile bombardment. Both methods exhibit comparable efficiency of stable transformation in wheat. The main limitations of the bombardment approach include fragmentation of the DNA during bombardment, the insertion of backbone vector DNA and the insertion of multiple genes [19]. The integration of unwanted vector DNA is undesirable, particularly in the present regulatory and consumer environment. Furthermore, multiple copies of the transgene at single loci and rearrangements of the transgenes have been frequently reported in wheat transformation studies using the microprojectile bombardment approach [20]. Numerous downstream breeding cycles are required to select out those transgenic plants with the best insertions and then to generate the homozygous lines required for the downstream breeding program and development of a commercial product. A large-scale comparison between *Agrobacterium*-mediated and

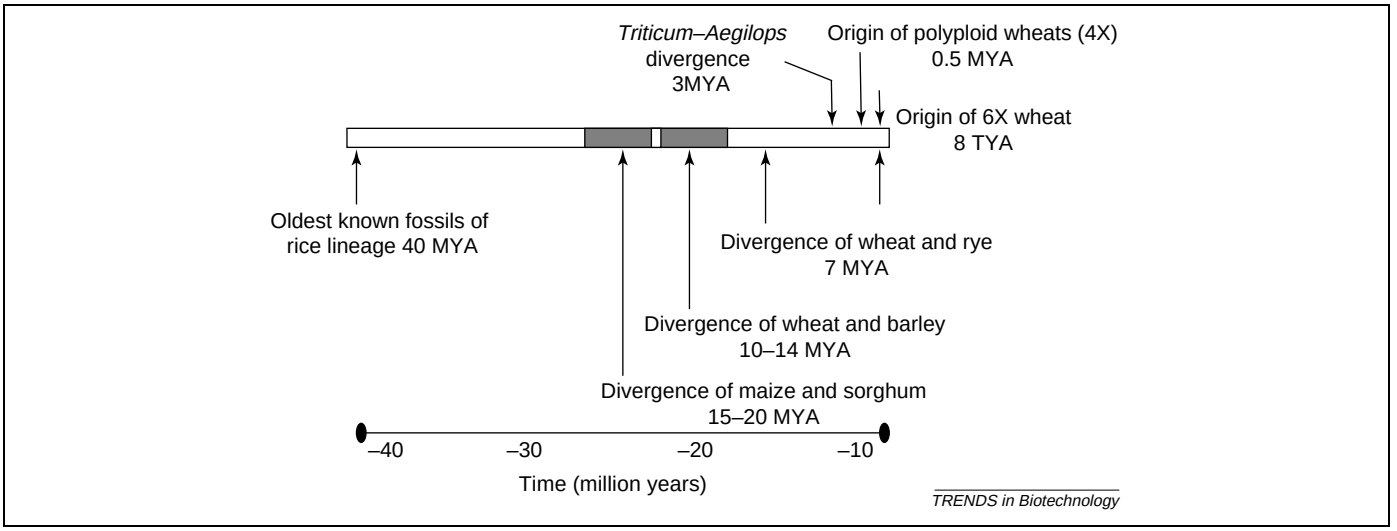

Figure 1. Schematic diagram depicting co-evolutionary history of major cereal crops [14]. Oldest known fossils of rice lineage date to 40-million-years-ago (MYA). Three major cereals, rice, maize and wheat, diverged from a common ancestor around same time. The diploid A, B and D progenitor species of wheat diverged from a common ancestor ~3 MYO, around the same time as humans diverged from apes. Modern humans originated ~200 000 years ago, which is virtually the same time as modern polyploid wheat originated by hybridization of diploid grass species.

microprojectile bombardment transformation methods indicated that the proportion of transgenic wheat plants carrying a single copy of the transgene and devoid of additional vector sequences was significantly higher for *Agrobacterium*-mediated transformation (>60%) than when using microprojectile bombardment (20%) [19]. In addition, the time required for the regeneration of transgenic plants was significantly lower when using *Agrobacterium*-mediated transformation [19], making this the system of choice for wheat (Figure 2).

Use of commercial varieties

The first study on the generation of fertile transformed wheat by biolistic bombardment describes selected callus lines being bombarded with DNA-coated particles followed by regeneration of viable plants from the transformed cells [18]. Gene delivery by *Agrobacterium*-mediated transformation was achieved more recently [20,21]; however, most of these studies used model spring-wheat varieties with inferior agronomic traits. The regeneration of transgenic commercial wheat varieties remains arduous. The incorporation of the introduced gene into the commercial varieties is achieved by backcrossing, which can take up to seven breeding cycles and, hence, lengthens the downstream breeding program in the development of a commercial product. So far, only hard red spring-wheat (cultivar Bobwhite), which is not a major type of wheat, carrying glyphosate herbicide resistance is at the commercial testing stage [22]. Although some success in developing protocols for locally adapted wheat germplasm has been reported [23–25], the development of a more robust system that is routinely applicable to commercial cultivars of different genetic backgrounds of locally adapted varieties is urgently needed.

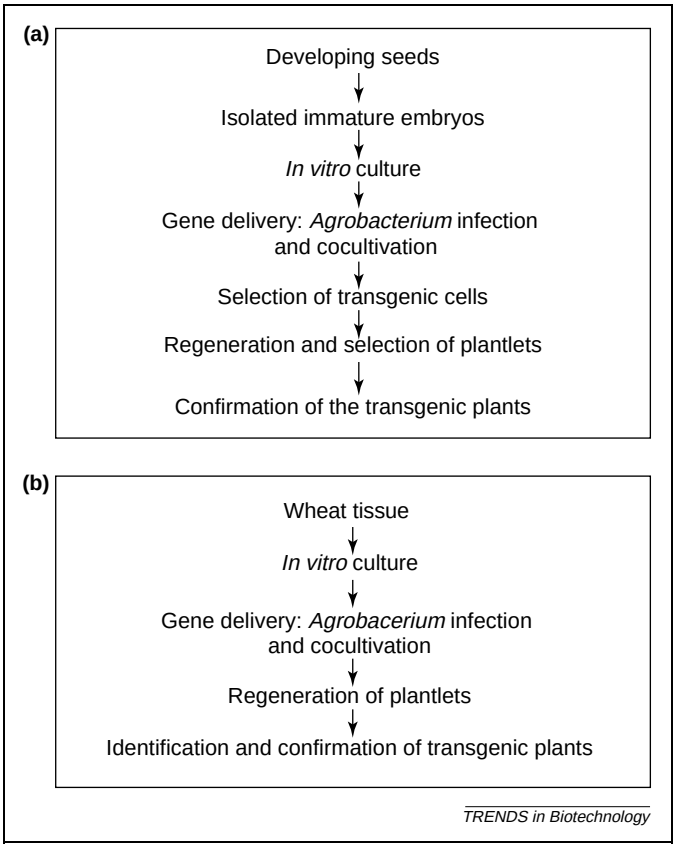

Figure 2. Genetic transformation of wheat. **(a)** Outline of currently used *Agrobacterium* method for wheat transformation in which a selectable marker gene and the gene of interest are introduced into the cells. Selection and regeneration of the transgenic cells are carried out in the presence of the selective agent (usually antibiotics), leading to production of transgenic plants carrying selectable marker gene in addition to the gene of interest. **(b)** Outline of a possible strategy (a hypothetical but a challenging goal) for the generation of marker-free wheat, where only the gene of interest is delivered into the cells using *Agrobacterium*, followed by the regeneration of plants. Identification of the transgenic status of the regenerated plants can be achieved by PCR.

Development of marker-free wheat

To date, the new commercialized crop varieties produced by genetic engineering contain a selectable marker in addition to the gene of interest. The selectable-marker genes have been considered essential for recovery of the transgenic plants during the transformation process but their continued presence raises public concerns and regulatory issues regarding the possible environmental impacts of horizontal transfer of resistance genes to other plants or pathogenic bacteria [26]. Furthermore, pyramiding the same cultivar with other desirable genes or traits requires new selectable markers each time, which limits the use of this technology because the repertoire of selectable markers applicable to a given crop is limited. Therefore, there is a need to develop technology that, ideally, does not require the use of a selectable marker in the first place, or else removes them efficiently after the selection of the transgenic plants.

Removal of selectable markers

Several studies have demonstrated various systems for the removal of the selectable markers: site-specific recombination [27], transposon-mediated elimination and cotransformation followed by segregation [28]. However, these methods have not been used for monocot crops (including wheat) because they are either difficult to implement or exhibit unacceptably low efficiency.

Recently, two approaches have been reported for excising the selectable marker in transgenic plants: the use of a chemical-regulated Cre/loxP system and heat-shock treatment. The chemical-regulated system was based on the use of β-estradiol in the culture medium, which resulted in 29% of regenerated rice plants being marker free with a single transgenic locus [29]. The use of β-estradiol in the culture medium saves time and labour because it removes the need for the regeneration of two types of transgenic lines followed by cross hybridization [30]. This also reduces the risk of genome rearrangement and the resulting aberrations in plants [31]; plants with a single transgenic locus exhibit more stable transgene expression compared with those with multiple transgenic loci.

The use of heat-shock treatment (three heat treatments at 42 °C for 2 hours) to remove a selectable marker using an autoexcision strategy (i.e. the Cre/lox system) is a promising and easy-to-adopt approach for the production of marker-free transgenic plants [32]. However, its use has only been demonstrated using tobacco, in which regeneration from a leaf explant is routine. This protocol uses leaf samples from primary transgenic plants, with subsequent

regeneration from the treated leaf explants, which might limit the application of the technique to wheat because plant regeneration from leaf explants has not been reported for monocot crops.

Marker-free transformation

Avoiding the use of a selectable marker during transformation has been successfully demonstrated for potatoes and cassava [33] using *Agrobacterium*-mediated transformation, although this approach has not been popular because it involves screening a large number of plants. However, it can be argued that PCR screening of plants during regeneration represents a small fraction of the overall cost of producing an improved cultivar. In addition, the availability of inexpensive robotic equipment to isolate DNA and set up a PCR from hundreds of plants improves the feasibility and simplicity of applying this approach to wheat. In fact, this marker-free, PCR-based approach has been successfully used to produce transgenic wheat by microprojectile bombardment [34], demonstrating the possibility of producing transgenic wheat without selection pressure.

Plant genes used as transformation markers

The use of plant-derived, herbicide-resistant forms of the genes encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS) are not desirable as selectable markers for wheat – the modified wheat can potentially become a weed. The selectable marker D-amino acid oxidase was recently used as a marker gene for the transformation of *Arabidopsis thaliana* [35]; however, given that *Arabidopsis thaliana* appears to lack endogenous activity of this enzyme, it remains to be seen whether this can be applied to wheat. Furthermore, overexpression of the *Arabidopsis* ABC transporter can confer kanamycin resistance during tobacco transformation, leading to the suggested use of this gene as a selectable marker for dicot crops [36]; however, hygromycin and geneticin are more widely used antibiotics for monocot crops, which limits the use of this gene for wheat transformation.

Recently, the use of phosphomannose isomerase in the selection of transgenic cells was demonstrated using durum wheat [37]. Selection efficiency of the commonly used *Bar* gene, where non transgenic cells die (negative selection) was compared with the gene encoding phosphomannose isomerase (*pmi*), where transformed cells have a metabolic advantage compared with untransformed cells (positive selection). Results showed that selection efficiency was much higher (90%) for plants using *pmi* than for those using the *Bar* gene (26%). Although the gene encoding phosphomannose isomerase used in the study was isolated from *E. coli*, the enzyme is common in plants such as soybean and other legumes, therefore raising the possibility of using this as a plant-derived selectable marker for wheat transformation.

Reduced backbone integration

Regulatory authorities require all introduced plasmid DNA, including backbone plasmid DNA, to be audited. Such auditing is difficult following microprojectile bombardment because this approach incorporates plasmid DNA fragments into the plant genome in a random fashion. Although DNA delivered by *Agrobacterium*-mediated transformation is supposedly restricted to the T-DNA of the plasmid, it is now well established that plasmid DNA is transferred from the binary vector to the plant [38,39]. The mechanism behind this transfer is not clearly understood but strategies for the commercialization of transgenic wheat require it to be free of this redundant backbone DNA. Strategies that use the genes encoding barnase and isopentenyl phosphotransferase have been applied, successfully, to screen for the transfer of backbone DNA [39,40].

Reducing the amount of intergenic DNA

Recent studies on the generation of marker-free tobacco plants (created using plastid transformation) and amylose-free potatoes have demonstrated the use of a few foreign gene elements during transformation. Although plastid transformation has not been successfully demonstrated for monocots, including rice, corn and wheat, the application of a similar approach to wheat deserves evaluation. In addition, plant transfer DNA (P-DNA) that resembles T-DNA has been used in transformation studies using potato and tomato [40]. Whether such an approach might be extended to monocot crops, including wheat, remains to be investigated.

Transgene silencing

Silencing of introduced genes is routinely observed in polyploid plant species such as wheat. Such transgene silencing has been observed in transgenic wheat plants generated by microprojectile bombardment [20,41], whereas wheat plants produced by *Agrobacterium*-mediated transformation seem to remain transcriptionally active [19], indicating the superiority of the latter route for use in wheat. In addition, the higher transformation efficiency and superior molecular characteristics of *Agrobacterium*-mediated transgenic events are supported by data from three years of field tests on a selected glyphosate-tolerant wheat line [42].

Recently, a microarray-based comparative analysis of gene expression profiles during grain development of wheat was attempted [43]: global, comparative gene-expression analysis is potentially a powerful tool in the assessment of transgenic plants. Gene expression profiles in developing seeds of untransformed wheat and a transgenic line containing endosperm-specific expression of an *Aspergillus fumigatus* phytase showed that increased phytase activity due to expression of the inserted gene resulted in unaltered overall gene expression patterns of the developing wheat seed.

Public debate and acceptance of the technology

The adaptation of genetically modified (GM) crops by farmers has been rapid; planting of GM crops by farmers has increased at a significant rate (double-digit rates per year) since their first introduction in 1996. This has led to an increase in the global GM crop area of more than 50-fold in the first decade of commercialization {C. James International Service for the Acquisition of Agri–biotech

Applications [ISAAA] Briefs 34–2005, *Global Status of Commercialized Biotech/GM Crops* 2005 (http://www.isaaa.org)}. However, public debate about GM crops has been intense, largely because of the absence of consumer benefits and the prospect of multinational companies controlling the main crops, leading to rejection of GM food in many European countries [44,45]. Environmental benefits, such as a between 60 and 80% reduction in pesticide use, resulting in improved health of the farming communities, due to the cultivation of *Bt* cotton are well established. Scientific assessment shows no unique health risks from GM crops, but opponents assert that consumption of transgenic plants is detrimental for human health – without supporting evidence and despite the fact that Americans have safely consumed food derived from GM crops more than nine years. It has been suggested that the real objections of the 'green' lobby groups are not based on scientific evidence but on fundamentalist ideology [46]. Much of the controversy regarding the use of GM crops is the extent to which these differ from the products of traditional breeding. Recently, Monsanto shelved herbicide-resistant wheat from further field trials owing to fear of consumer rejection: the product is useful to some farmers, although this is a not one of the main wheat variety grown, but offers no direct benefit to consumers. However, the company is developing products that they hope will appeal to consumers, such as healthier oils and longer-lasting produce [22].

There has been much discussion in the scientific literature regarding the development of a framework for designing transgenic crops [26,47] and the associated regulatory processes [48]. The main objection to GM crops is concern about the introduction of genetic material from distantly related organisms, for example, the insertion of animal DNA into plants. Therefore, to enable differentiation of the origins of the DNA, a categorization of the engineered crop to clarify that it contains recombinant material from the same taxonomical family (intragenic) or contains genes designed in the laboratory (xenogenic) has been proposed [47]. Such distinction might help in maintaining the cultural values, perception and traditional identity of the product, leading to increased public acceptance.

In 2005 a significant milestone was reached when four new countries joined the list of GM crop-growing countries, bringing the total number to 21 {C. James ISAAA Briefs 34–2005 *Global Status of Commercialized Biotech/GM Crops* 2005 [http://www.isaaa.org]}. Of the four new countries, three were EU countries, Portugal, France, and the Czech Republic. In addition, several hundred farmers in Iran grew GM rice containing the *Bt* gene, commercially, for the first time on approximately four-thousand-hectares. Rice is the staple food for more than half the population of the world and is grown by 250 million farmers. Thus, the commercialization of GM rice will contribute significantly towards the reduction of poverty, hunger and malnutrition. Increased adoption of GM rice is significant not only for the rice growing and consuming countries but also for acceptance of all GM crops.

Ongoing and consistent implementation of GM crops is indicative of productivity enhancement, with environmental and economic gains being realized by both growers and consumers alike. It is envisioned that delivery of improved traits for wheat, as outlined in Box 1, will have a profound impact on food security and our society in general.

Box 1. Examples of transgenic traits that might help to address the needs of a growing population.

Stress tolerance

Abiotic stress

Drought tolerance
Salt tolerance
Oxidative-stress tolerance
Improved aluminium, boron, cold and heat tolerance

Biotic stress

Disease resistance – fungal, viral and bacterial pathogens
Pest resistance – insect and nematode

Agronomic traits

Herbicide tolerance
Improved efficiency of water use
Hybrids

Quality characters

Improved grain quality
Improved nutritional quality – reduced phytate content, improved macronutrients and essential micronutrients content, and improved amino acid composition
Modified gluten character for celiac disease sufferers
Speciality wheats with health promoting nutriceuticals in grains

Conclusions and future perspectives

The projected increase in the worldwide demand for food and the shortfall in grain yields require a fully integrated approach to crop improvement for sustained agricultural productivity. Gene transformation is one of the vital tools available to modern plant breeding for the development of crop plants with desired traits. Most traits introduced by gene transformation are dominant and can therefore be transferred from one plant to another as a single locus. The choice of gene-transformation methodology and the design of transgenic crops require careful planning that addresses the public and regulatory concerns influencing the acceptance of GM crops (Box 2). Nonetheless, gene transformation

Box 2. Key issues

- Wheat is a major staple crop that is grown worldwide. The predicted worldwide population growth requires doubling of its production from the current level by 2050.
- Plant breeding continues to improve wheat production but is limited by the gene pool required for developing varieties for abiotic and biotic stress tolerance and the value-added characteristics to improve human health.
- Recombinant methods have the potential to meet the future demands but require robust transformation technology that is directly applicable to established and adapted commercial cultivars.
- Public perception and social pressure demands transgenic crops to be free from selectable antibiotic and/or herbicide-resistant gene markers and other redundant intergenic recombinant DNA.
- Recent progress in transformation technology clearly demonstrates that it is possible to engineer wheat with desired traits.
- Improved wheat varieties would have a significant impact on food security, human health and nutrition.

Box 3. Main issues and current challenges for wheat genetic transformation.

Issues:
Genotype dependent
Time and resource consuming
- Need for developing seeds as a starting material
- Selection and regeneration of plants

Use of antibiotic and/or herbicide resistance as a selectable marker
Transgene silencing

Current challenges:
Direct transformation of commercial cultivars
Rapid regeneration protocol
Marker-free regenerated plants
Single-gene insertion with minimal or no extra foreign DNA

complements breeding outcomes because it provides the potential to transfer specific traits into selected genotypes without affecting their desirable genetic background. To date, only a small number of agronomically important genes have been introduced into wheat. However, the development of new transformation strategies and advances in genomic research provide a massive potential for the genetic engineering of wheat with enhanced traits (Box 1). The future of the genetic engineering of wheat lies in the successful development of a targeted gene-delivery system that does not require a selectable marker and is genotype independent (Figure 2b; Box 3), making the fast-track improvement of commercial breeding lines and varieties a reality.

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