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Review article

Current progress and challenges in crop genetic transformation

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ABSTRACT

Plant transformation remains the most sought-after technology for functional genomics and crop genetic improvement, especially for introducing specific new traits and to modify or recombine already existing traits. Along with many other agricultural technologies, the global production of genetically engineered crops has steadily grown since they were first introduced 25 years ago. Since the first transfer of DNA into plant cells using *Agrobacterium tumefaciens*, different transformation methods have enabled rapid advances in molecular breeding approaches to bring crop varieties with novel traits to the market that would be difficult or not possible to achieve with conventional breeding methods. Today, transformation to produce genetically engineered crops is the fastest and most widely adopted technology in agriculture. The rapidly increasing number of sequenced plant genomes and information from functional genomics data to understand gene function, together with novel gene cloning and tissue culture methods, is further accelerating crop improvement and trait development. These advances are welcome and needed to make crops more resilient to climate change and to secure their yield for feeding the increasing human population. Despite the success, transformation remains a bottleneck because many plant species and crop genotypes are recalcitrant to established tissue culture and regeneration conditions, or they show poor transformability. Improvements are possible using morphogenetic transcriptional regulators, but their broader applicability remains to be tested. Advances in genome editing techniques and direct, non-tissue culture-based transformation methods offer alternative approaches to enhance varietal development in other recalcitrant crops. Here, we review recent developments in plant transformation and regeneration, and discuss opportunities for new breeding technologies in agriculture.

1. Plant and crop genetic engineering address global agricultural challenges

Since the first announcements by three research groups of the genetic transformation of plants using the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid at the Miami Winter Symposium in 1983 (O'Brien, 1983), genetically engineered (GE) crops are now being grown in 41 countries (English and Kayleen, 2020) and represent an increasing percentage of global crop production. In the U.S. alone where GE crops were first grown in 1996, more than 75 % of total corn, cotton, soybean and sugar beet crops to date are now genetically engineered for herbicide and insect resistance (Economic Research Service, United States Department of Agriculture, 2020). In 2020, the global market for GE crops and seeds is estimated at nearly US$ 28 Billion and is expected to reach US$ 45 Billion by 2027 (Global Industry Analysts, 2020). The Green Revolution in the 1960′s and 1970′s gave farmers higher-yielding and more resistant semi-dwarf crop varieties (Pingali, 2012), which prevented food famines that occurred in the past, such as the Bengal famine in 1943 or the Irish potato famine from 1845 to 1849, both of which were caused by pathogens. Since then, genomics-assisted breeding and GE technologies have further improved crop yield and nutritional qualities to feed the growing human population. But there is concern that yield growth is stagnating or even collapsing for the key global crops maize, rice, soybean and wheat, which is likely exacerbated by climate change (Challinor et al., 2014). These developments represent a serious challenge for doubling global crop production by 2050 to meet agricultural demands (Lobell et al., 2011; Ray et al., 2013, 2012). Today, we are at the brink of a second Green Revolution that will bring new crop traits and digital technologies to agriculture and farmers (Aubry, 2019; Marvin, 2018; Shepherd et al., 2020), of which plant and crop transformation is an essential part. This is not too soon because providing food security for 9.6 billion people by 2050 we need to increase crop productivity while reducing the agricultural footprint on the environment, prevent crop losses caused by pathogens and adverse

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climatic conditions, and enhance the nutritional qualities of crops to improve human health. This also requires a broader acceptance of GE crops and their products.

2. Genetic transformation accelerates crop trait development for agricultural production

Conventional and new breeding techniques (NBTs, see below) together with advanced transformation technologies are arguably at the core of achieving global food security (Landry and Mitter, 2019). Often plant transformation is referred to as 'genetic engineering', but for the purpose of this review we consider plant transformation as the process of creating a single or binary vector with appropriate gene(s) in expression cassettes, direct delivery or indirect transfer by in planta transformation of a T-DNA or other DNA construct containing an expression cassette into a plant cell, and regeneration of the transformed plant cell into a transgenic plants (Newell, 2000). Plant transformation employs a wide range of methods aimed at either the expression of one or more introduced gene (transgenes), silencing the expression of one or more endogenous genes, or modifying the activity or function of one or more endogenous genes.

The first successful regeneration of a transgenic plant from transformed plant cells expressing a bacterial gene encoding neomycin phosphotransferase dates back to 1983 (Barton et al., 1983; reviewed in Somssich, 2019). This was followed by the report of a leaf disc method for *Agrobacterium*-mediated transformation and regeneration of plants that was successful for petunia, tobacco and tomato (Horsch et al., 1985). Since then plant transformation methods were continuously improved and expanded, and new direct transformation methods have been developed (Table 1). This facilitated the analysis of basic plant gene functions and development of new traits in crops. Today, as many as 525 different transgenic events across 32 crops are approved for cultivation (Kumar et al., 2020). But challenges remain for plant transformation, which continues to be costly, time-consuming, and difficult for transformation-recalcitrant plant species and crop genotypes.

2.1. Improvement of the plant transformation process

Whole genome sequences facilitate target discovery and construction of transformation vectors. Parallel to the development of transformation technologies, automated and low-cost long-read DNA sequencing technologies together with effective genome assemblers (Li and Harkess, 2018) now make whole-genome sequencing of plant and crop genomes a routine affair. To date, several diploid and polyploid plant genome assemblies have been reported, including from globally important crops such as rice (Goff et al., 2002; Yu et al., 2002), maize (Schnable et al., 2009), sorghum (Paterson et al., 2009), potato (Xu et al., 2011), banana (D'Hont et al., 2012), tomato (The Tomato Genome Consortium, 2012), barley (Mascher et al., 2017), wheat (Appels et al., 2018) and cassava (Kuon et al., 2019), or the complex octoploid genome of the desiccation tolerant 'resurrection' plant *Xerophyta viscosa* (Costa et al., 2017). Emerging insights into the structure of pan-genomes already reported for barley (Jayakodi et al., 2020), maize (Hirsch et al., 2014), wheat (Walkowiak et al., 2020) and other plants (Khan et al., 2020) now increases our understanding of the plasticity of plant genomes, thereby providing new opportunities for both conventional breeding and targeted genetic engineering of crops. Challenges remain, however, because high-quality reference genomes, genome annotations or information on allelic variation are still missing for many crop species. Progress is being made in improving the assembly of complex genomes using BAC-based assembly strategies (Visendi et al., 2016), resolving repetitive sequences with long reads (Du and Liang, 2019), or using multiple reference genomes (Kolmogorov et al., 2018) and strategies for assembling polyploid genome sequences (Kyriakidou et al., 2018). The application of chromosome conformation capture technology (Hi-C) has also greatly aided the assembly of high-quality genomes of model plants (e.g., (Wang et al., 2015) and complex homozygous and heterozygous crop genomes (e.g., (Kuon et al., 2019; Mascher et al., 2017). There are also increasing efforts to establish reference genomes for each of the 'orphan crops' to accelerate their improvement through breeding and transgenic technologies, which could help to improve their nutritional qualities to address micronutrient malnutrition ('hidden hunger') in developing and under-developed countries (Jamnadass et al., 2020; Van Der Straeten et al., 2020).

While rapid genome sequencing technologies produce large amount of sequence information at the gene level, 'new genes' still have to be experimentally validated for their function predicted from *in silico* analysis (Bouchez and Höfte, 1998). It is possible to infer gene function from transcriptome, protein interactome, metabolome, genetic interaction and 3D structure data (Rhee and Mutwil, 2014). Transgenic plants in which genes can be manipulated greatly accelerate the validation of gene function in the context of complex gene networks at different plant developmental stages and/or in relation to external stimuli (Kochetov and Shumny, 2017). Transgenic plants can also help to understand the activity of the promoter or the coding sequences.

Table 1
First or early reports of successful development of select transgenic plants and crops.

Plant and crop type	Common name (scientific name)	References
Cereal crops	Barley (*Hordeum vulgare*)	(Deng et al., 1990)
	Maize (*Zea mays*)	(Rhodes et al., 1988)
	Rice (*Oryza sativa*)	(Shimamoto et al., 1989)
	Sorghum (*Sorghum bicolor* L. Moench)	(Casas et al., 1993)
	Soybean (*Glycine max*)	(Hinchee et al., 1988; McCabe et al., 1988)
	Wheat (*Triticum aestivum*)	(Deng et al., 1990)
Root and tuber crops	Cassava (*Manihot esculenta* Crantz)	(Li et al., 1996)
	Potato (*Solanum tuberosum*)	(An et al., 1986)
	Sweet potato (*Ipomoeu batatas* (L.) Lam.,)	(Otani et al., 1993)
	Yam (*Dioscorea rotundata*)	(Nyaboga et al., 2014)
Fruit and vegetable crops	Apple (*Malus domestica* Borkh)	(James et al., 1989)
	Banana and Plantain (*Musa* spp.)	(Sági et al., 1995)
	Cauliflower (*Brassica oleracea* L. var. Botrytis	(David and Tempé, 1988)
	Citrus (*Citrus reticulata* Blanco)	(HIDAKA et al., 1990)
	Papaya (*Carica papaya* L.)	(Fitch et al., 1990)
	Pea (*Pisum sativum* L.).	(Puonti-Kaerlas et al., 1990)
	Plum (*Prunus domestica* L.)	(Mante et al., 1991)
	Strawberry (*Fragaria ananassa*)	(James et al., 1990)
	Tomato (*Lycopersicon esculentum*)	(Horsch et al., 1985)
	Watermelons (*Citrullus lanatus*)	(Choi et al., 1994)
Oil and fiber crops	Canola (*Brassica napus* L)	(Pua et al., 1987)
	Mustard (*Brassica juncea*)	(Pua et al., 1987)
	Oil palm (*Elaeis guineensis*)	(Kadir and Parveez, 2000)
	Peanut (*Arachis hypogaea* L. cv. New Mexico Valencia A)	(Mathews et al., 1990)
	Sunflower (*Helianthus annuus* L.)	(Schrammeijer et al., 1990)
Woody plant species	Populus hybrid NC-5339 (*Populus alba x grandidentata*)	(Fillatti et al., 1987)
	Walnut (*Juglans regia* L.)	(McGranahan et al., 1988)
Commercial crops	Cannabis (*Cannabis sativa* L.)	(MacKinnon et al., 2001)
	Cotton (*Gossypium hirsutum* L.)	(Umbeck et al., 1987)
	Sugarcane (*Saccharum officinarum* L.)	(Bower and Birch, 1992)
	Sugarbeet (*Beta vulgaris*)	(Harpster et al., 1988)
Others	Petunia	(Horsch et al., 1985)
	Tobacco	(HORSCH et al., 1984)
	Nicotiana (Nicotiana tabacum & Nicotiana plumbaginifolia)	(De Block et al., 1984)
	Flaveria plants	(Martineau et al., 1989)

3. Building informed expression cassettes from characterized components

Generating transgenic plants involves the construction of gene expression cassettes that contain promoter and coding sequences in a choice of DNA vectors, the selection of a suitable transformation method for a tissue to be transformed, as well as effective selection and regeneration methods. The success of establishing functional transgenic plants lies in designing expression cassettes that have adequate or optimal expression levels of transgenes in specific tissues and at appropriate developmental timepoints or in environmental conditions. A typical expression cassette consists of the coding sequences for one or more genes of interest controlled by appropriate regulatory sequences, i.e. promoters with known expression patterns (constitutive, inducible, or tissue-specific) and a transcription terminator. The extent to which coding sequences may affect transcription of transgenes in the context of expression is difficult to predict and must be experimentally determined. Therefore, the choice of necessary and essential regulatory components for transgene expression is pivotal and equally important as the choice of coding sequences for the construction of expression cassettes. Identification of either 'novel' coding sequences or regulatory elements that determine the activity of the promoters remains quite challenging and time-consuming.

To date, available genome sequences and transcriptome data are often used for the identification of promoter regions. Most of the selected promoter regions are active in transgenic plants, but the level of transgene activity often depends on the length of selected promoter regions. Event-dependent variation in transgene expression levels are not uncommon as the result of the positional effect of transgene integration (Hernandez-Garcia and Finer, 2014). In addition, except for a few promoters, most of them do not show the same activity in other crop species as found in the plant species from which they were isolated. Therefore, it might be necessary to experimentally validate the activity of selected promoters in the genetic background of the target plant or crop that would be transformed. The currently available choice of reported tissue-specific or condition-responsive promoters across crop species is still quite limited and represents a bottleneck that constrains rapid progress in genetic engineering of crop plants (Basso et al., 2020), especially when engineering a metabolic pathway that involves many genes. To alleviate this limitation to some extent, the role of functionally defined DNA sequence modules is becoming increasingly important for designing and constructing synthetic promoters. Such designed promoters containing functional genetic modules that determine their strength and specificity (either constitutive, spatio-temporal, inducible, or even tissue-specific; Hernandez-Garcia and Finer, 2014) are gaining increasing importance in transformation strategies (Ali and Kim, 2019; Dey et al., 2015).

4. Innovative cloning kits facilitate the construction of expression cassettes

Recent improvements in assembling large DNA fragments with a predefined number and arrangement of transgenes, together with the increased efficiency of DNA cloning methods, has simplified and accelerated the construction of multigene expression cassettes. Many methods such as the iterative cloning system GoldenBraid for the standardized assembly of reusable genetic modules (Sarrion-Perdigones et al., 2011), the Golden Gate Modular Cloning (MoClo) kit (Binder et al., 2014; Weber et al., 2011), the GreenGate system (Lampropoulos et al., 2013), the Gibson assembly method first used for DNA library construction (Thomas et al., 2015), and the well-known Gateway cloning method (Karimi et al., 2007) now offer researchers choices that best fit their strategies for the construction of transformation cassettes. The relative ease of stacking genes can facilitate the engineering of metabolic pathways in plants and microorganisms. Among the available cloning methods, the GoldenBraid2.0 (GB2.0) and Golden Gate MoClo assembly kits are most widely used for this purpose (Patron et al., 2015).

Both the GoldenBraid2.0 (GB2.0) and Golden Gate MoClo assembly methods use TypeIIS restriction enzymes, which recognize non-palindromic and asymmetric DNA sequences motifs and cleave outside of their recognition site, leaving pre-defined single-strand DNA sequence overhangs. Of the many TypeIIS restriction enzymes, Golden Gate MoClo assembly uses *Bpi*I (*Bbs*I) for constructing basic transcription units and *Bsa*I and *Esp*3I for assembling and combining other transcriptional units to stack several genes a transgene expression cassette. For example, using the MoClo assembly strategy, eleven transgene expression units consisting of 44 individual basic modules could be assembled in only three successive cloning steps (Weber et al., 2011). The GoldenBraid2.0 (GB2.0) assembly method is quite similar to Golden Gate MoClo but uses *Bpi*I(BbsI) and *Bsm*BI restriction enzymes for assembling the transgene expression units. An important prerequisite for both assembly strategies is that all modules that are used for construction of transgene expression cassettes do not contain DNA sequences that can be recognized and cleaved by the above-mentioned TypeIIS enzymes. Both GoldenBraid2.0 (GB2.0) and Golden Gate MoClo assembly kits use destination-level vectors with known antibiotic resistance to combine all DNA parts and also provide an adapted binary vector for *Agrobacterium tumefaciens*-mediated transformation. The assembly of the standard DNA parts is largely error-proof because it does not require PCR and gel purification steps.

5. Different methods are available for transfer of expression cassettes into plant cells

Transgenic approaches are widely used to validate gene function in model species such as Arabidopsis, tobacco or rice, and they have found broad application in the development of new or improvement of established traits in several crops (Kumar et al., 2020). Transformation of most plants requires the availability of suitable target tissues (explants) or cells and their handling under aseptic conditions, an efficient method to introduce DNA into these target tissues or cells, and protocols for the selection and regeneration of transgenic plants. The success and efficiency of establishing somatic embryos from explants for transformation and regeneration depend on the nature of the explants and their treatments (Cheng et al., 2003, 1997; Grzyb and Mikuła, 2019; Prakash and Gurumurthi, 2009). Several types of explants, generally obtained from young tissues (leaf, meristem, hypocotyl), are used for somatic embryogenesis and transformations. Somatic embryos derived from somatic/vegetative cells, are quite comparable embryos developed from to zygotic cells. Somatic embryogenesis is the process by which the embryo develops from a somatic cell, which eventually regenerate into a plant under appropriate conditions(Guan et al., 2016). Various explants from seedlings (roots, hypocotyls, cotyledons), young leaves excised from in vitro-grown plants, apical meristems, flower buds, peduncles, anthers, and immature embryos are used (Tomiczak et al., 2019). Somatic embryos are comparable to zygotic embryos and display similar developmental stages - global, heart, torpedo, and cotyledon (Garcia et al., 2019). Friable -embryogenic callus (FEC) are small clusters of numerous spherical embryogenic units that are light yellow and are derived from derived from sub culturing somatic embryos (Bull et al., 2009; Ma et al., 2015).

Both, somatic embryos and FEC can be transformed and used for generate transgenic plants, but the major difference is that somatic embryos can give rise to unstable /chimeric plants (Saelim et al., 2009) while FEC give rise to plantlets from a single cell and thus reduces the chance of generating a chimeric plants (Bull et al., 2009). In some instances, gametophytic cells or embryonic microspore cultures have been transformed via inoculation with Agrobacterium. This approach is particularly attractive for generating homozygous transgenic plants via whole genome duplication in initially haploid regenerants, but will not be discussed further in this review. Not all plant species or different genotypes of a particular plant species can be easily established in tissue

culture for somatic embryo production and transformation. Thus, after almost 40 years since the first report of plant transformation using Agrobacterium, the production of transgenic plants for specific plant genotypes or farmer-preferred crop varieties is still complex and the biological reason for genotype-dependent transformation remains largely unexplained. Recent advances of using transcription factors that function as plant morphogenetic regulators to improve transformation and regeneration capacity may prove useful in advancing less genotype-dependent transformation strategies (see below; Kausch et al., 2019).

To date, many direct and indirect methods are available for delivering transgene expression cassettes into plant cells and tissues (Saifi et al., 2020). Table 2 lists the methods that have been employed for generating GE crops now approved for commercial production.

Among all plant transformation methods, particle bombardment and *Agrobacterium*-mediated transformation are most widely used for producing transgenic plants (Gelvin, 2003; Twyman and Christou, 2004). Both methods have their own advantages. Transgenic plants obtained by *Agrobacterium*-mediated transformation generally have a more stable expression of the transgenes and a higher frequency of intact single copies of the introduced expression cassette in independent transformation events. Transgenic plants obtained from particle bombardment typically contain a higher average copy number of the expression cassette (Dai et al., 2001; Travella et al., 2005). It is important to note that DNA sequence fragments of the T-DNA plasmid used in *Agrobacterium*-mediated transformation have been detected in transgenic maize (Shou et al., 2004), although such non-intended T-DNA insertion events seem to be infrequent. At times, both particle bombardment and *Agrobacterium*-mediated transformation methods are used in combination to overcome *in vitro* tissue culture constraints of regeneration-recalcitrant plants (Gurusaravanan et al., 2020; Paes de Melo et al., 2020). A selection of T-DNA binary vector systems and disarmed *Agrobacterium* strains are now frequently used for plant transformation(De Saeger et al., 2020; Hwang et al., 2017; Lee and Gelvin, 2008). Some of the *Agrobacterium* strains have higher transformation efficiency (Komari, 1989). Other *Agrobacterium* strains have been modified by either addition of genes or mutation of existing genes in its genome, which has successfully improved the efficiency of plant transformation (Hansen et al., 1994; Nonaka et al., 2019, 2008a, 2008b).

Several improved and reproducible transformation protocols using *Agrobacterium* and biolistic delivery of DNA in plants have been developed for numerous plant and crop species along with a range of explants (Altpeter et al., 2016; Rustgi and Hong, 2020), including crops such as cassava and rice that are important for small-scale farmers (Bull et al., 2009; Hiei and Komari, 2008; Nyaboga et al., 2013), as well as fruit crops and emerging model species (Osakabe et al., 2018; Nguyen et al., 2020). These methods are continuously adapted and improved in many model and non-model plants as well as economically important crops (see https://bio-protocol.org, Plant transformation). A schematic representation of key steps in constructing expression cassettes and *Agrobacterium*-mediated plant transformation is shown in Fig. 1 for cassava as example.

Transformation methods are also increasingly used to deliver CRISPR/Cas enzymes for functional genomics approaches and rapid crop trait improvement through genome editing (Hickey et al., 2019). The introduction of CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR associated protein), has opened up a new system for genome editing at a precise site (Jinek et al., 2012). This system is also greatly exploited for its high efficiency, ease of use, and accuracy and thus finds application in genome editing in both, simple and complex genomes (Manghwar et al., 2019). The usage of CRISPR/Cas for editing crop genome for improving specific traits is reported in across many crops for improving yield, improving crop performance under abiotic and biotic stress and improving the nutritional Content and quality of the crops (Bao et al., 2019).

The primary requirement for genome editing is the delivery and expression of CRISPR/Cas in the recipient cell. The delivery of CRISPR/Cas components is done either by stable-integration or transient expression of the foreign DNA. The stable-integration of CRISPR/Cas components are mainly delivered by Agrobacterium-mediated (Chandrasekaran et al., 2016; Soyk et al., 2017; Zhang et al., 2018), and particle bombardment (Shi et al., 2017; Wang et al., 2014) and this relies on tissue culture or non-tissue culture (de novo induced meristem (Shi et al., 2017)) based genetic transformation.

Other delivery methods, such as PEG-mediated transfer of CRISPR/Cas components are either stably expressed or transiently expressed (Andersson et al., 2017). The transient expression of the CRISPR/Cas components is performed by delivering pre-assembled Cas9-sgRNAribonucleoproteins (RNPs) in protoplasts cultures for creation of DNA free edited plants (Woo et al., 2015). In addition, to PEG-mediated, particle bombardment method is also used to deliver RNPs (Banakar et al., 2020; Svitashev et al., 2016). Other methods include electroporation (Lee et al., 2020) and lipofection (Liu et al., 2020) have also been used for delivering RNPs.

Table 2
Methods used for trait development and improvement in different crops that are currently approved for commercial production.

Method of crop transformation	No. of crop events approved for release
Conventional breeding: cross-hybridization and selection involving transgenic donor(s)	273 (e.g., bean, canola, cotton, maize, rice, soybean, etc.)
Agrobacterium tumefaciens-mediated plant transformation	195 (e.g., alfalfa, apple, canola, cotton, maize, potato, tomato, etc.)
Microparticle bombardment of plant cells or tissue	45 (e.g., canola, cotton, maize, soybean, etc.)
Chemically-mediated introduction into protoplasts and regeneration	2 (maize)
Direct DNA transfer system	2 (rice)
Electroporation	2 (maize)
Pollen-tube pathway	2 (cotton)
Aerosol Beam Injection	1 (maize)
Whiskers-mediated plant transformation	1 (maize)
Agrobacterium rhizogenes-mediated plant transformation	1 (canola)

Source: https://www.isaaa.org/gmapprovaldatabase/traitintrolist/default.asp

6. Direct transformation methods avoid potential tissue culture-dependent plant regeneration bottlenecks

Traditionally, GE crop development requires tissue culture techniques that are quite complex and involve isolating cells or specialized tissues, growing these cells or tissues in defined conditions and establishing protocols for their transformation with *Agrobacterium* or other approaches, and finally regeneration of the cells or tissues into transformed plants. This is often a time-consuming and labor-intensive process that requires specialized experimental skills. At times, the regenerated plants can exhibit desirable or undesirable trait variations that arise from mutations or genome instability during tissue culture (Fossi et al., 2019), which may increase or reduce the value of the transformed plants. As discussed above, successful and efficient regeneration of transformed plants is often influenced by the type of tissue explant or its genotype, especially for different crop varieties (Ahmed et al., 2018; Mahto et al., 2018). Therefore, transformation methods that avoid tissue culture would be very beneficial. One of the most widely used direct transformation method is the floral dip protocol developed for *Arabidopsis thaliana* (Clough and Bent, 1998). This method has been adopted in other crops with some modifications such as applying vacuum (Chhikara et al., 2012; Lu and Kang, 2008), floral spraying (Chung et al., 2000), and floral injection (Sharada et al., 2017). These methods seem to be most efficient for members of the Brassicaceae species, but they have also been reported for other crops, e.g., wheat (Zale et al.,

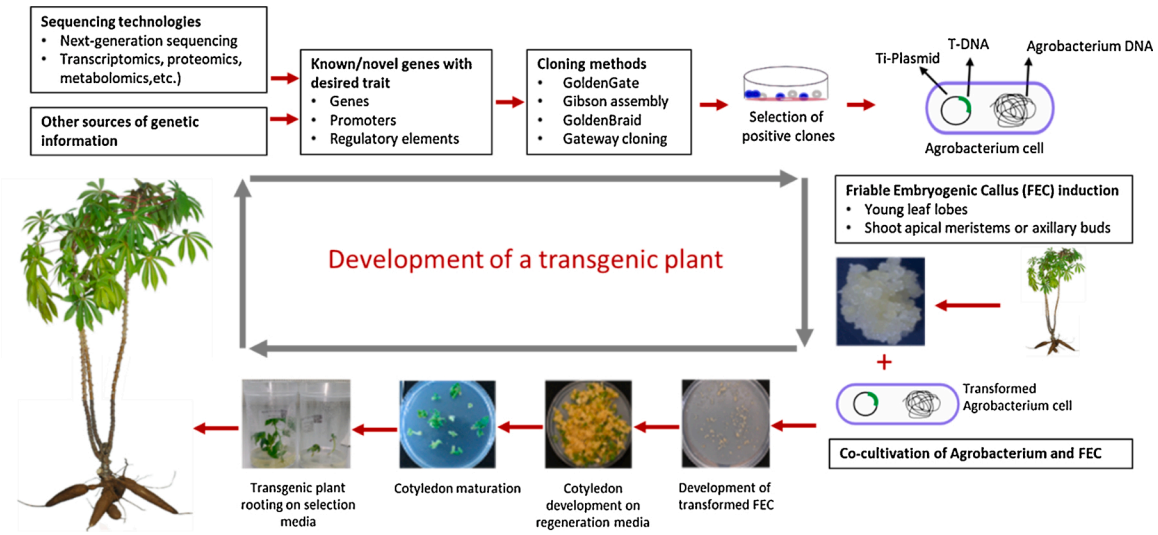

Fig. 1. Plant transformation involves the construction of DNA vectors with assembled gene expression cassettes based on knowledge about the function of genes and regulatory DNA sequences that control their expression in the transgenic plants or crops. These expression cassettes are transferred into Ti-DNA plasmids contained in Agrobacterium, which transfers the T-DNA into the plant cell where the expression cassette is integrated into the nuclear genome. Other methods can be used to introduce expression cassettes into the plant cell. Cassava is an example of a crop that involves a series of complex tissue culture steps (shown in clockwise order from the right) to produce friable embryogenic callus (FEC) can be transformed with Agrobacterium (Bull et al., 2009). Several subsequent tissue culture steps are required to select cotyledonary embryos that can be regenerated into transformed plantlets and grown into full plants for testing of introduced traits in the greenhouse or field.

2009), *Setaria viridis* (Saha and Blumwald, 2016), rice (Ratanasut et al., 2017), and tomato (Yasmeen et al., 2009). In addition to the inflorescence, other explants such as seeds (Karthik et al., 2018; Subramanyam et al., 2013) and cotyledons (Kalbande and Patil, 2016) have also been used for direct transformation approaches. However, considering the longstanding concern about problems associated with plant transformation by inoculating florets with *Agrobacterium* (Langridge et al., 1992), reports using this method must be critically assessed for stable transgenesis and unambiguous evidence of heritable transgenicity.

More recently, tissue culture-independent transformation strategies involving the *de novo* induction of meristems (Maher et al., 2020), pollen, and nanoparticles to deliver DNA or RNA (Lv et al., 2020) have been reported for the production of GE plants. The *de novo* induction of meristems, termed Fast-TrACC (fast-treated *Agrobacterium* co-culture), is a convenient method for the ectopic delivery of developmental regulators (DRs) to seedlings to induce the growth of meristems that can be transformed, which has been used successfully to generate gene-edited plants (Maher et al., 2020). In maize, transformed pollen grains have been used to deliver transgenes for the production of GE plants (Yang et al., 2017). Similar methods of delivering transgene constructs via pollen were successfully adopted in many other crops (Ali et al., 2015). Together, direct DNA delivery methods can overcome tissue culture or genotype-dependent transformation bottlenecks and therefore could be adapted to many transformation-recalcitrant crops if heritable transgenicity can be rigorously demonstrated.

The Direct DNA transfer system involve the uptake of T-DNA (endocytosis) from the surrounding solutions via the transient pores created in the plasma membranes in plants cells (Ozyigit, 2020; Saul and Potrykus, 1990). The creation of the transient pores is stimulated either by chemical (polyethylene glycol (PEG), calcium phosphate precipitation) or by electroporation (applying an electric pulse) (Chen et al., 2006; Vorobiev and Lebovka, 2016). The Direct DNA transfer method depends on factors such as the chemical concentration or type of electrical pulse applied. In plants, though chemical and electroporation methods are successfully applied in protoplasts for generating transgenic plants but electroporation method has also been applied to transform other cell types (stomata guard cell, anther, microspore, zygote, mature and/or immature embryo, mesophyll, nodal meristems (Ozyigit, 2020).

Pollen-tube pathway is also a direct DNA mediated transformation method that circumvents the tissue culture requirements and can be successfully used in crop varieties that have limitation of regenerating transformed cells. As the name suggest the T-DNA (genomic DNA, plasmid, single gene) is microinjected into pollen tube sacs for integration of T-DNA into the genome and for generating transgenic lines (Wang et al., 2013; Yang et al., 2009, 2011).

Aerosol Beam Injection This is also a direct DNA transfer and is quite similar to particle bombardment and microinjection. Here the target tissues are injected from a continuous liquid microstream (aerosol beam) and thus provides an advantage over the bombardment and microinjection (Que et al., 2019; Yadava et al., 2017).

Whisker-mediated DNA delivery employs silicon carbide whiskers that are micro-fibers 10–80 mm long and 0.6 mm in diameter to transform plant cells. Here the callus/cells is placed into a buffer containing plasmid DNA along with the silicon fibers, which is then vortexed for DNA to be delivered (Frame et al., 1994; Petolino et al., 2000).

Agrobacterium rhizogenes-mediated plant transformation: A similar method as that of the Agrobacerium tumefaciens except that Ri plamsids from A. rhizogenes are used for vectors for delivering the T-DNA (Suzuki et al., 2009). Agrobacterium rhizogenes-mediated plant transformation is quite used in transformation of hairy root cultures for enhanced production of metabolites (Gutierrez-Valdes et al., 2020; Ismail et al., 2019)

7. Challenges in selecting plant transformants

Selectable marker genes are required for the identification of transformed plantlets during the regeneration of cells, tissues, or shoots that contain delivered DNA constructs. They are classified as positive or negative selectable marker genes depending on their function in transformed cells. Negative selectable marker genes encode either enzymes that confer resistance to specific antibiotics (such as kanamycin, paramycin, hygromycin, etc.) or herbicides (phosphinothricin, glyphosate) (Sundar and Sakthivel, 2008). Positive selectable marker genes encode enzymes that allow transformed cells to metabolize specific substrates (phosphomannose, sugar alcohol, amino acid analogs) that cannot be metabolized by plant cells (Aragão and Brasileiro, 2002). Additionally, genes encoding enzymes or proteins that produce visible signals (e.g.,

color or fluorescence) serve as screen-able markers, also termed 'reporter genes' encoding e.g. green fluorescence protein (GFP; Harper et al., 1999; Kaeppler et al., 2001), FAST protein (Fluorescence-Accumulating-Seed Technology; Shimada et al., 2011), DsRed protein (Lu and Kang, 2008), or enzymes for anthocyanin pigment production (Dutt et al., 2018; Kanizay et al., 2016). These genes are used for screening rather than selecting transformants, which is the final step in the transformation process. In some countries, the presence of selectable marker genes in GE crops is of regulatory and societal concern, especially when they contain genes for enzymes providing resistance to antibiotics that are still used in medical treatments.

Consequently, methods to produce transformed plants that are free of selectable marker genes are now receiving more attention. Such transformed plants can be generated either by avoiding selectable marker genes in the transformation process and using PCR for screening of transformed plants, co-transformation of the selectable marker gene and the gene(s) of interest on separate Ti-plasmids and subsequently segregating the selectable marker gene from the trait-conferring gene by genetic crosses (Kapusi et al., 2013; Miller et al., 2002; Singh et al., 2017) or removal of the selectable marker gene from existing transformants (Goldsbrough et al., 1993; Murovec et al., 2018; Zhao et al., 2019; Zubko et al., 2000). The production of marker-free transgenic plants either by removal or non-usage of selectable marker genes should help to address the concerns related to gene flow from transgenic plants to non-GE crops or wild relatives, and also reduce the metabolic burden from unwanted marker gene expression on the plants and could enhance the public acceptance of transgenic crops (Yau and Stewart, 2013).

The strategies such use of Cre/lox recombination system (or also known as induction-excision strategy) (Borrill, 2020; Dale and Ow, 1991; Sreekala et al., 2005; Wang et al., 2020), Ac-Ds transposon system (Cotsaftis et al., 2002) or use of bacteriophage-λ attachment (attP) regions (Zubko et al., 2000) or an auto-excision system (Du et al., 2019), are implemented for removal of selectable marker gene.

In the Cre/lox recombination systems, the T-DNA region carries the Cre recombinase, lox site along with the gene of interest. The Lox sites are located on either side of the region (selectable marker) that is to be removed from the transgenic cassette. The Cre recombinase expressed under an inducible promoter recognizes and cleaves at the lox sites and thus releasing the selectable marker (Sreekala et al., 2005).

In Ac-Ds transposon system, the T-DNA carries transposase and the transposases recognizing sites along with the selectable marker gene and gene of interest. The location of the transposases recognizing sites (either adjacent to the selectable marker gene or the gene of interest), then is selected based on gene segregation in the next generation (Cotsaftis et al., 2002).

Alternatives are co-transformation methods using genes encoding fluorescence marker proteins such as GFP (Yang et al., 2019) or the β-glucuronidase enzyme (GUS; Chen et al., 2020; Rosellini, 2012).

7.1. Genome location-independent and stable expression of transgenes

Stable and reproducible transgene expression is an important aspect of GE crop development. The expression of a transgene is influenced by many factors. Variable expression of the transgene is often the result of its transcriptional or post-transcriptional inactivation (Fagard and Vaucheret, 2000; Rajeev Kumar et al., 2015), the genome ploidy level of the plant expressing the transgene (Finn et al., 2011; Mittelsten Scheid et al., 1996), or the chromosomal location into which the DNA construct has integrated (Finn et al., 2011; Matzke et al., 2002). Integration of multiple copies of a transgene can also facilitate gene silencing (Rajeev Kumar et al., 2015; Tang et al., 2007). Variable transgene expression in independently transformed plants of the same genetic background that were produced under identical conditions is not uncommon. Such variability can be minimized by generating a large number of independent transgenic events and pre-screening these events for stable and comparable transcription of the transgene, similar protein expression levels from the transgene, and avoiding events with multiple transgene insertions, as it is routinely done for the development of commercial GE crop products (Bakó et al., 2013; Privalle et al., 2012). In standard transformation methods such as biolistic and *Agrobacterium*-mediated DNA construct delivery it is generally not possible to control the T-DNA integration site or the number of integration events. Random transgene insertion events occasionally disrupt the activity of endogenous genes because selection of transgenic events tends to shift the recovery of T-DNA insertions into gene-rich or transcriptionally active regions of chromatin (Kim and Gelvin, 2007). Such events are not acceptable for the regulatory approval of GE crop products, which makes the evaluation of transgenic events for commercial use labor- and cost-intensive, typically involving multi-year risk assessments in field trials (Slot et al., 2018). Several transformation strategies have been developed to minimize variation among independent transgenic lines and thus to reduce the cost of commercial development of GE crops. Pre-evaluated plant promoters with optimized regulatory sequences along with appropriate terminators are often used to reduce variation of transgene expression. Matrix attachment regions (MARs) are included in DNA expression cassettes to stabilize gene expression and minimize transgene silencing (Allen et al., 2000; Butaye et al., 2005; Dolgova and Dolgov, 2019). MARs or 'insulators' enhance gene autonomy and prevent promoters in transcription units from affecting each other in multigene cassettes, and also minimize the position effect on transgenes in the plant genome (Zhao et al., 2019). Together, these strategies help to retain the tissue-specific expression of candidate transgenes and enhance their stable expression in transformed plants.

Ideally, a transgene should be inserted into the plant genome at a predefined target DNA sequence (landing pad) that has been characterized for gene expression stability and copy number integration, as well as any effect of transgene insertions on the otherwise equivalent performance of the transgenic plant or agronomic performance to the transgenic crop (Danilo et al., 2018; Kirchhoff et al., 2020). Methods to insert transgene DNA constructs at specific sites in the genome are already established in mammalian tissue culture cell lines using recombinase-mediated insertion of heterologous DNA (Gaidukov et al., 2018) and in yeast cells using synthetic DNA landing pads that have been engineered in the genome (Bourgeois et al., 2018). In plants, designed zinc-finger nucleases that create targeted double-strand DNA breaks were first reported for site-specific transgene integration (Shukla et al., 2009). Recent strategies using DNA nucleases or CRISPR-Cas9 enzymes promise rapid advances in targeting transgene insertions to defined landing pads in plants and crops for reproducible transgene expression and transgene stacking (Gao et al., 2020; Petolino and Kumar, 2016; Zhao et al., 2019). Such strategies might also prove useful for editing or replacing existing transgenes as well as endogenous genes to accelerate specific trait development or trait performance improvements of crop plants.

7.2. Continuing challenges for plant transformation and GE trait development

7.2.1. Generating transformable callus from diverse crops and genotypes remains a bottleneck

The genome modification and editing approaches discussed above, also referred to a new breeding techniques (NBTs), are revolutionizing the rapid development of novel crop traits (Schaart et al., 2016) but also raise new regulatory questions in countries that consider plants or crops obtained with NBTs involving genome editing as GE events (Jorasch, 2020). Realizing the full potential of NBTs remains challenging, however, because efficient and less genotype-dependent plant transformation and regeneration are still bottlenecks. To date, the majority of the GE crops in the market are limited to a few varieties for which callus induction, transformation and plant regeneration are efficient and robust transformation protocols have been established. Exploiting new crop varieties, especially among orphan crops that should be promoted

because they are important for food security of smallholder farmers and consumers in developing countries (Tadele, 2019), is often limited because they are recalcitrant to genetic transformation and funds are lacking to establish them in tissue culture. Explant tissues of recalcitrant species usually turn brown, which is caused by the accumulation and oxidation of phenolic compounds induced by wounding stress (Dixon and Paiva, 1995; Dreger et al., 2019). It is difficult to induce and maintain callus tissue from such explants, and substantial experimental effort is required to modify tissue culture protocols before callus tissue can be obtained that is amenable to transformation. Recent efforts of increasing transformation rates in new varieties of major crops have been successful by expressing the genes for the maize BABY BOOM (BBM) and WUSCHEL2 (WUS2) transcription factors in different maize genotypes (Lowe et al., 2018, 2016) and recalcitrant sorghum varieties (Nelson-Vasilchik et al., 2018). Expression of the Arabidopsis GRF5 (AtGRF5) transcription factor has similarly increased transformation rates in both monocot and dicot crops such as sugar beet, canola, maize, soybean, and sunflower (Kong et al., 2020) that are otherwise not easily transformable using standard transformation methods. A novel fusion protein of the wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its co-factor GRF-INTERACTING FACTOR 1 (GIF1), when expressed in wheat, triticale and rice, increased the efficiency and speed of plant regeneration (Debernardi et al., 2020). While these growth regulators are useful for improving transformation and regeneration efficiencies in crops that are transformable in principle, they do not solve the problem for many plant and crop species, or crop genotypes of obtaining culturable and transformable callus tissue from explants in the first place, which thus remains a serious bottleneck for crop improvement that needs to be addressed. Sometimes modifying the concentration or composition of hormonal growth regulators is successful for achieving transformation of recalcitrant genotypes (Lentz et al., 2018; Maheshwari et al., 2011), but more systematic efforts are required to understand what makes a plant or genotype transformable or not. In addition, use of L-cysteine and/or thiol compounds (Na-thiosulfate, dithiothreitol (DTT) during co-cultivation have shown to significantly increase the transformation efficiency in soybean (Olhoft et al., 2003). Also, *A. tumefaciens* is known interact with many host factors and are also know to modify the expression of defense-related genes, production of reactive oxygen species (ROS) and manipulate the hormonal levels (Gelvin, 2009; Pitzschke, 2013). These changes induces browning and cell death in many crop plants and so, affects the recovery of transgenic plants (Khanna et al., 2004, 2007; Wroblewski et al., 2005). Suppression of the host defense mechanisms has shown an increase in both, transient expression as well as stable transformation and regeneration of the transformed cells (Khanna et al., 2007; Wang et al., 2018; Zhang et al., 2013).

7.2.2. Plant transformation centers in developing countries are needed for improvement of national and local crops

Developing and under-developed countries are confronted with many challenges – poverty, population health, and poor economic performance. Not all, but some challenges can be addressed by adopting GE crop technologies, especially from the perspective of smallholder farmers (Adenle, 2011). Adoption of GE crops results in increased yield with reduced agricultural input (Klümper and Qaim, 2014), consumers are willing to pay more for crops with health benefits (De Steur et al., 2017), and nutritionally-fortified GE crops can improve consumer health (Qaim, 2010; Van Der Straeten et al., 2020). Areas under GE crop cultivation are increasing in many industrialized countries, while the adoption and cultivation of GE crops is still limited in developing and under-developed countries (especially in the African Union). Efforts are now underway in these countries to establish public research centers for GE crop development. This requires financial and trained personnel resources for research and development, policies and strategies for dealing with the constraints of intellectual property rights and regulations for product development (Huesing et al., 2016), easy norms for public-private (both national and international) partnerships, and transfer of established plant transformation methods to research laboratory and national transformation facilities in developing countries (Bull et al., 2011; Chetty et al., 2013; Nyaboga et al., 2013). In parallel, consumers need to be made aware of the benefits from GE crops and establishing agriculture biotechnologies in their own countries to empower crop improvements.

7.2.3. Standardized and globally accepted regulatory procedures are needed for GE crop testing and approval

As a pre-requisite for the commercial agricultural use of any GE (and non-GE) crop, the new variety along with its (non-GE) sibling has to be tested for its agricultural performance in open field trials at multiple locations before it can be released as a new variety. The majority of the concerns for open field trials with GE crops, especially in Europe, revolve around protecting human and animal health, as well as the environment, from perceived impacts of the GE traits (Sparrow, 2010). Unintended gene flow from GE plants to non-GE plants (Sanvido et al., 2007; Tsatsakis et al., 2017), development of secondary pest resistance (Catarino et al., 2015), or potential allergenicity of proteins expressed in GE plants (Mishra and Arora, 2017), are some of the concerns that must be addressed before the release of new GE crops for open field trials and commercialization. Review and approval of applications for open field trials are quite complex, although some countries have established detailed guidelines for GE field trials (Slot et al., 2018). Also, the approval time for conducting GE field trials varies across countries. The approval for the release of a GE crop as a new variety is often a tedious, lengthy and expensive process involving many government and non-government organizations (NGOs). This affects progress in GE crop research and development in public research institutions that do not have the financial resources for bringing GE crops to market. Therefore, it would be preferable to establish uniform and consistent guidelines for GE field trials and approval processes for the release of new GE crop varieties that are globally followed and accepted.

7.2.4. The acceptance of GE crops remains a contentious but solvable issue

Although famers are rapidly accepting GE crops for cultivation, the major obstacle towards the public acceptance of GE crops and their products are consumer concerns about health impacts. However, most consumers lack a comprehensive understanding of the methods that are used today for crop improvements (Lucht, 2015). This is being exploited by many NGOs opposing GE crops, who promote false or misleading information about GE technologies in rich societies without consideration how this affects farmers in developing countries who could benefit from GE crops (Paarlberg, 2014). While scientists can produce robust and convincing data about the benefits and risks of using GE crops in agricultural production, there also has to be a political will in governments of explaining to their citizens how GE crops and NBTs along with precise crop production technologies can reduce the agricultural footprint on the environment and slow the rapid progression of climate change. More investments for the development of new and improved plant transformation technologies will also facilitate crop improvements to maintain yield stability and secure a safe food supply – a win-win situation for both consumers and the environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this pape.

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