Front. Agr. Sci. Eng. 2017, **4**(1): 5–27
DOI : 10.15302/J-FASE-2017131
Available online at http://engineering.cae.cn

REVIEW

Plant genetic engineering and genetically modified crop breeding: history and current status

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Abstract This review charts the major developments in the genetic manipulation of plant cells that have taken place since the first gene transfer experiments using Ti plasmids in 1983. Tremendous progress has been made in both our scientific understanding and technological capabilities since the first genetically modified (GM) crops were developed with single gene resistances to herbicides, insects, viruses, and the silencing of undesirable genes. Despite opposition in some parts of the world, the area planted with first generation GM crops has grown from 1.7 Mhm^2 in 1996 to 179.7 Mhm^2 in 2015. The toolkit available for genetic modification has expanded greatly since 1996 and recently Nobel Laureates have called on Greenpeace to end their blanket opposition, and plant scientists have urged that consideration be given to the benefits of GM crops based on actual evidence. It is now possible to use GM to breed new crop cultivars resistant to a much wider range of pests and diseases, and to produce crops better able to adapt to climate change. The advent of new CRISPR-based technologies makes it possible to contemplate a much wider range of improvements based on transfer of new metabolic pathways and traits to improve nutritional quality, with a much greater degree of precision. Use of GM, sometimes in conjunction with other approaches, offers great opportunities for improving food quality, safety, and security in a changing world.

Keywords plant transformation, transgene, genetic modification

Received October 20, 2016; accepted December 2, 2016

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1 Introduction

Advances in molecular genetics and genetic modification are bringing revolutionary changes to society. One of the key aspects is the use of biotechnology to modify the genomes of plants in new ways to help provide enhanced value to support sustainable production of food, materials, energy, and even therapeutic components. In this article we review the various scientific developments in genetic modification that underpinned the production of transgenic plants, defined as those with genomes altered by the transfer of a gene or genes from another species, and genetically modified (GM) plants, defined as having genetic material altered in a way that does not occur naturally through fertilization and/or natural recombination. We also discuss some of the advantages, and problems associated with the first generation of GM crops, and review recent developments that offer new opportunities for designer breeding to improve crop plants and make them more suitable for our needs.

Genetic transformation (changing the genetic characteristics of an organism by introducing a specific piece of DNA from another source) began with research on bacteria by Griffith[1] and Avery et al.[2]. Griffith showed that genetic characteristics could be transferred artificially by an agent transferred from heat-inactivated cells to live ones and that the change was heritable. Avery et al. demonstrated that the chemical component responsible for determining the genetic characteristics was DNA. These discoveries started a revolution in genetics because they provided methods for testing and assigning a genetic function to a specific piece of DNA, and for transferring gene segments between bacteria in a laboratory to change their genetic characteristics in a controlled manner. The

discovery of the structure of DNA by Watson and Crick in 1953, the demonstration that the genetic code was (almost) universal, and the advent of gene cloning and DNA sequencing in the 1970s[3–6] led to an explosion of activity in the new field of molecular genetics and was a spur to the development of new methods for genetic transformation of animals and plants. It is no exaggeration to say that this has revolutionized our knowledge of the functioning of eukaryotic cells, and our understanding and capabilities in medicine, agriculture, and plant and animal breeding, and now underpins major segments of the pharmaceutical and biotechnological industries.

Over the past 20 years, GM crops have undergone an explosive increase from proof of concept to a key component of crop improvement. From 1996 to 2015 the total area of GM crops grown in the world increased from 1.7 to 179.7 Mhm^2, or roughly 13.2% of the arable land in the world[7]. Major GM crops grown include alfalfa, canola, cotton, maize, papaya, potato, soybean, squash, and sugar beet. Of the 28 countries growing "biotech crops" in 2015, the top 10 countries accounted for 89% of the GM crops planted[7] (Table 1). The USA tops this list with 70.9 Mhm^2, and now about 107 Mhm^2 of GM crops are grown in Africa, Asia and South America. This review charts the development of methods for genetic transformation of plants, the advantages and disadvantages of the different approaches used, and their applications in the breeding and production of GM crops worldwide.

2 Common tools for DNA transfer and detection

The idea of transferring DNA to plants in order to test gene function and breed new plants without going through normal sexual processes has been at the forefront of research in plant molecular biology for 50 years. Early claims that soaking seeds or whole plants in DNA could lead to a heritable change in their genetic characteristics were met with scepticism, and various approaches have been developed since the 1980s to improve the efficiency of delivery, integration and expression of exogenous DNA. After being added to a plant cell, DNA must also be integrated into the host's genetic material in order for it to be inherited through cell divisions and regeneration of a plant. It is also necessary to utilize appropriate gene promoters that are effective at controlling expression of exogenous genes and a suitable selectable marker gene so that cells with the exogenous DNA can easily be recognized and selected. Regeneration of transformed plants from cells receiving the added DNA depends on the totipotency (the ability of a single cell to regenerate into a complete new individual) of plant cells, which was demonstrated in plants in the 1950s by FC Steward and others[8].

2.1 Reporter genes

A reporter gene confers an easily detectable phenotype on a recipient organism, and is often attached to a regulatory sequence or a gene of interest to monitor transgenic events or gene expression. Although more than 50 reporter genes have been described[9], only a few of them including *gusA*, green fluorescent protein (*GPF*) and its wavelength shifted variants, and luciferase (*LUC*), have been used extensively for plant research and crop development. While reporters are very useful for fundamental experiments, however, some consumer groups would prefer not to have reporter genes in foods, and this is actually no longer necessary.

2.1.1 *gusA* gene

The bacterial *gusA* (formally *uidA*) gene encoding a β-glucuronidase (GUS, E.C. 3.2.1.31) is one of the most commonly used reporter genes in plants. The GUS enzyme is able to hydrolyze a wide variety of β-glucuronides. One of the most widely used substrates is 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, which is colorless but oxidized

Table 1 Top ten countries growing GM crops in 2015

Country	Area/Mhm^2	Crops
USA	70.9	Maize, soybean, cotton, canola, sugar, beet, alfalfa, papaya, squash, potato
Brazil	44.2	Soybean, maize, cotton
Argentina	24.5	Soybean, maize, cotton
India	11.6	Cotton
Canada	11.0	Canola, maize, soybean, sugar beet
China	3.7	Cotton, papaya, poplar
Paraguay	3.6	Soybean, maize, cotton
Pakistan	2.9	Cotton
South Africa	2.3	Maize, soybean, cotton
Uruguay	1.4	Soybean, maize

Note: Data from James, 2015, Crop Biotech Update, Special Edition 13 April 2016; http://www.isaaa.org/kc/cropbiotechupdate/specialedition/2016/2016-04-13-cbu.html.

by GUS to form an indigo blue chromogenic precipitate, which can easily be detected quantitatively or qualitatively in plant cells or extracts. As a reporter in transgenic plant research, *gusA* has many advantages, such as a very low endogenous GUS-like activity, lack of toxicity to plants, and high stability and activity in translational fusions with other proteins. Moreover, the GUS protein is rapidly degraded under the conditions in the animal stomach, and is nontoxic to humans and animals[10], meaning from a toxicological viewpoint GUS transgenic plants and their products are safe for the environment and consumers.

2.1.2 Fluorescent protein genes

Green fluorescent protein (GFP) was originally isolated from the jellyfish *Aequorea aequorea* in 1962[11]. The wildtype GFP has a major absorbance peak at 395 nm and smaller one at 470 nm, and emits green light at 509 nm. However, this particular form of GFP was not suitable as a reporter because of its low brightness, photobleaching and improper folding at 37°C[12,13]. To overcome these limitations, several improved GFP isoforms with enhanced fluorescence were generated by mutagenesis[12–14]. In addition to these "optimizing" mutations, several mutants emitting blue, cyan, and yellow light were also developed[15]. Nowadays, these fluorescent proteins have become powerful reporters to analyze gene expression and determine protein localization in different cells and subcellular compartments (Fig. 1) and also for studying protein–protein interactions in living cells[16–18]. For their contribution to the discovery and development of GFP, Osamu Shimomura, Martin Chalfie and Roger Y. Tsien won the Nobel Prize in Chemistry in 2008.

2.1.3 Luciferase gene

Another well-known reporter encodes luciferase (LUC) which catalyzes the ATP-dependent oxidative decarboxylation of luciferin[19]. David Ow and colleagues expressed a cDNA copy of the firefly luciferase mRNA under the control of the CaMV 35S promoter (see below) in carrot protoplasts and transgenic tobacco using a Ti plasmid vector and the plants produced light when supplied with the substrate[20]. Importantly, LUC loses activity rapidly in the presence of luciferin, with a half-life of about 2–3 h[21], thus its activity more accurately reflect transgene expression (a combination of mRNA transcription, translation and degradation rates) than does the activity of GUS or GFP reporters, whose proteins are more stable[21]. Using two different luciferases, Renilla and firefly luciferase, McNabb et al.[22] developed a novel dual luciferase assay system for rapid assessment of gene expression. When one luciferase was placed under the control of a constitutively expressed promoter, it provides an internal control for

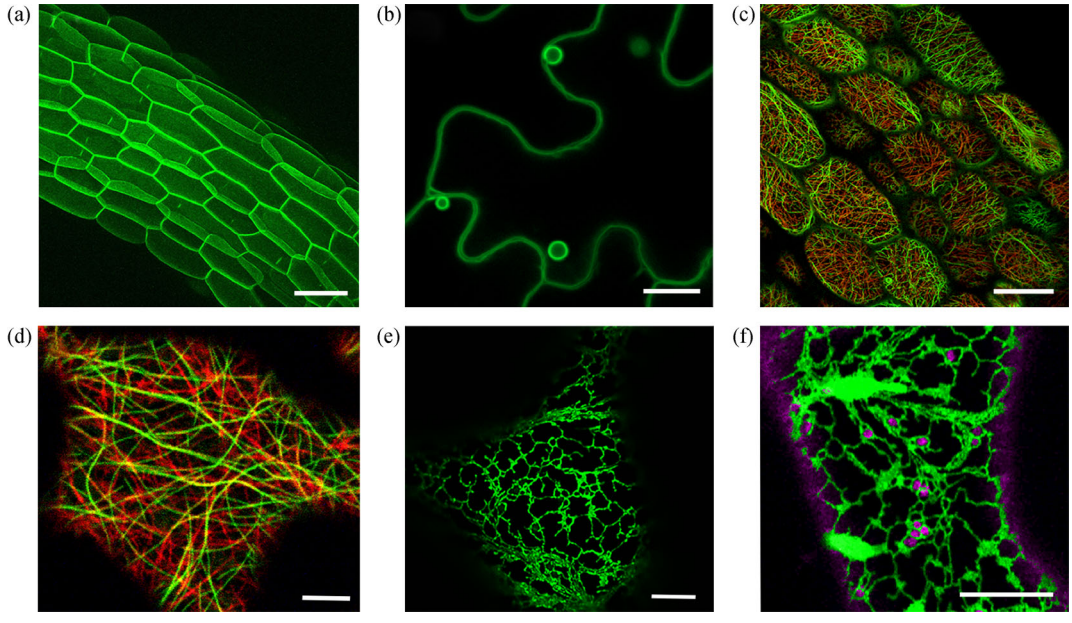

Fig. 1 Confocal laser scanning microscopy of plant cells labeled with various fluorescent protein constructs targeted to different organelles. (a) Reconstruction from a Z series of an *Arabidopsis* seedling hypocotyl labeled with a plasma membrane marker, LTI6B::GFP. Bar = 20 μm; (b) leaf epidermal cells of a tobacco leaf labeled with a vacuolar membrane marker BobTIP::GFP. Bar = 10 μm; (c) *Arabidopsis* hypocotyl cells expressing a GFP labeled tubulin for microtubules (green filaments) and an mCherry (red) fluorescent protein attached to an actin binding protein (red filaments). Bar = 20 μm; (d) higher magnification of an *Arabidopsis* leaf cell expressing microtubule and actin labels as in graph (c). Bar = 5 μm; (e) *Arabidopsis* leaf epidermal cells expressing the endoplasmic reticulum marker GFP-HDEL. Bar = 5 μm; (f) double labeled *Arabidopsis* leaf epidermal cell expressing the endoplasmic reticulum marker GFP-HDEL (green) and the Golgi body marker ST-mRFP (magenta). Bar = 10 μm. (a), (c) and (d) are courtesies of Joe McKenna, (b) and (f) are courtesies of Chris Hawes, and (e) is courtesy of Verena Kriechbaumer, Oxford Brookes University, UK.

normalizing the expression of the second luciferase under the control of the regulatory elements being studied.

2.1.4 Selectable marker genes

Selectable marker genes are pivotal to plant genetic transformation and are present in the vector along with the target gene. The commonly used selectable markers are antibiotic- or herbicide-resistance genes, which confer resistance to toxicity of antibiotic or herbicide.

The most widely used antibiotic selectable marker genes are neomycin phosphotransferase II (*nptII*) and hygromycin phosphotransferase (*hpt*), both from *Escherichia coli*. The *nptII* gene, encoding a neomycin phosphotransferase which inactivates aminoglycoside antibiotics such as kanamycin, neomycin, and geneticin by phosphorylation[23]. Among these aminoglycoside antibiotics, kanamycin and geneticin are mostly used as the selective agents. Although widely used in a diverse range of plant species, kanamycin is ineffective for selecting several gramineae and legumes, for example *Setaria italica* and *Brassica napus* and the *hpt* gene is more suitable than *nptII* for the selection of these plant species. The product of the *hpt* gene inactivates hygromycin B, an aminocyclitol antibiotic interfering with protein synthesis[24]. Besides *nptII* and *hpt*, other antibiotic marker genes such as that encoding chloramphenicol acetyl transferease have also been reported, but these genes are not as commonly used in transformation research.

The bialaphos resistance (*bar*) gene is a classic herbicide selectable marker, which inactivates the herbicide phosphinothricin by converting it into the acetylated form[25]. To date, the *bar* gene has been used successfully in many plant genetic transformation systems, including *Arabidopsis thaliana*, rice, tobacco and tomato[25–27].

The selectable chemicals mentioned above usually inhibit regeneration of transformed cells, and thereby decrease transformation frequency. To overcome this limitation, some selectable marker systems based on genes promoting shoot formation were developed and used successfully in plant transformation[28]. A good example is the isopentyltransferase (*ipt*) gene, which catalyzes the first step in cytokinin biosynthesis[29]. Unlike the commonly used antibiotic- or herbicide-resistance markers, the *ipt* gene can promote plant regeneration without the use of selective agents. Thus, this type of selectable markers has great potential to improve the transformation frequency of recalcitrant species.

2.2 Gene promoters

The promoters used in plant genetic transformation largely determine the expression profile of the added gene, i.e., when, where and how much of the mRNA product is produced. Gene promoters are traditionally divided into three categories: constitutive, induced and tissue-specific promoters. Constitutive promoters are active in all cells at all times, while the induced and tissue-specific promoters are active only in specific tissues or in the presence of external signals that induce expression.

2.2.1 Constitutive promoters

The importance of the cauliflower mosaic virus (CaMV) 35S promoter was first highlighted by Covey et al.[30]. This promoter controls the synthesis of the 35S major transcript of the CaMV virus[31,32] and is the most commonly used constitutive promoter in plant genetic transformation. The typical CaMV 35S promoter is a 352-bp fragment spanning nucleotides -343 to $+9$[33]. A duplication of the -343 to -90 fragment can enhance transcriptional activity greatly[34], thus it is commonly used in plant expression vectors. Although widely used, the CaMV 35S promoter has some limitations such as its weaker activity in monocots and in some cell types, for example, pollen and embryo sac[35,36]. To overcome this problem, several monocot-derived promoters have been isolated and evaluated including *OsAct1*[37] and *OsAct2*[38], *OsCc1*[39], *OsTubA1*[40], *rubi3*[41], *RUBQ1* and *RUBQ2*[42] from rice, and *ZmUbi1*[43] from maize. Compared to the CaMV 35S promoter, these promoters are highly active in monocot crops, and thus more suitable for regulating gene expression in cereals[44]. In addition to the promoters mentioned above, the nopaline synthase (*nos*) and the octopine synthase (*OCS*) gene promoters, derived from the *Agrobacterium* Ti plasmid also featured extensively in plant gene transformation, to drive the expression of selectable marker genes[45,46]. Since they are used, either together or separately, in many transformed organisms, these constitutive promoter sequences are often chosen as markers to identify genetically modified plants.

2.2.2 Tissue-specific/inducible promoters and inducible gene expression systems

In many cases, constitutive overexpression of a target gene may cause unexpected phenotypic changes such as reduced growth or even lethality. To avoid these problems, many different tissue-specific or inducible promoters have been identified and developed as inducible gene expression systems. Tissue-specific promoters are divided into four main categories according to the tissues where they are expressed, i.e., root, leaf, floral organ and seed/fruit specific promoters. They have been well summarized previously[47,48], and will not be reviewed here. Gene expression driven by tissue-specific promoters is largely dependent on the plant developmental stage, whereas inducible promoters can be precisely turned on by an external stimulus. In plants, various stresses, both biotic and abiotic, could induce expression of many genes. These

stress-inducible promoters are valuable for the development of resistant crops in which the resistance genes are turned on only under stress conditions.

Over the last three decades, several systems for induction of transgene expression in plants have been developed based on inducible promoters[49–52]. The most widely used inducible systems are those induced by heterologous hormones because they do not activate endogenous plant pathways. For example, Zuo et al.[51] developed a human estrogen receptor-based system consisting of the bacterial repressor LexA (X), the human estrogen receptor (ER or E) and the acidic transcriptional activation domain from VP16 (V). The XVE system is tightly regulated and highly induced by 17β-estradiol. In the presence of 17β-estradiol, XVE binds to the *LexA* domain, thus activating the transcription of the downstream target gene. This system has been successfully used for gene overexpression in various plant species[51,53–55], and has also been employed successfully for fundamental research on plant gene action. Other inducible plant gene expression systems, for example those using dexamethasone[50], ethanol[56], and tetracycline[57] as inducers, have also been developed and used successfully. Although these systems are powerful tools for basic research in gene function analysis and genetic manipulation, they are not suitable for agricultural use, because their inducer is expensive and/or difficult to apply. Saijo and Nagasawa[49] developed an effective copper inducible system and used it successfully to control flowering time. Compared to the above inducers, copper, which is a constituent of some fungicides, is readily taken up by plants, inexpensive and easy to apply in the field, thus could be used in both functional genomics and agribiotechnology.

3 Development of plant transformation systems

The early history and developments of plant cell culture has been documented by Sussex[8]. Subsequently, a range of different plant parts have been tested for suitability for DNA transfer experiments, including seeds, whole plants, plant segments, callus cultures and protoplasts. The generation and use of plant protoplasts (cells from which the walls have been stripped by treatment with enzymes) was pioneered at Nottingham University by Cocking in 1961[58]. Protoplasts proved to be very suitable for the introduction of DNA or viruses and a further important development was the introduction of polyethylene glycol to greatly enhance the uptake of exogenous DNA by protoplasts[59]. In addition to adding DNA fragments, it proved possible to transfer chromosomes between cells of different species by protoplast fusion, forming cell hybrids known as cybrids. Since protoplasts from many species of plants are able to synthesize a new cell wall, divide and regenerate into new plants, this made it possible to overcome incompatibility barriers encountered during normal sexual reproduction and produce new plants by a process called somatic hybridization. A detailed review of these developments is beyond the scope of this article but for an account of the history and applications of protoplast technology the reader is referred to the review by Davey et al.[60]

An alternative method for introducing DNA was developed, including firing DNA-coated gold or tungsten microprojectiles into plant cells (a process known as biolistics) using cells in callus culture, or discs cut from stems or leaves, using a gene gun[61]. Another successful method was the use of small silicon carbide whiskers for treatment of cultured cells in suspension culture to generate transient holes in the plasma membranes to aid passage of DNA into the cells[62]. The proceedings of an EMBO workshop on gene transfer to plants gives a fascinating account of a range of approaches that have been tested[63]. The biolistics and whiskers methods have the advantage that with appropriate selection they can be used to introduce DNA into either the nuclear or the plastid (chloroplast) genomes[64].

Perhaps the most widespread and successful method for plant genetic transformation, however, is derived from a naturally-occurring bacterium, *Agrobacterium tumefaciens*, and its relative *Agrobacterium rhizogenes*. During the 1970s and 1980s it was discovered that *Agrobacterium* causes tumors by transferring a specific DNA fragment (the T-DNA) from a tumor-inducing (Ti) plasmid to the nucleus of a wounded plant cell, where it becomes incorporated into one or more of the chromosomes. Once this was understood and the Ti plasmid had been genetically characterized, it was obvious that specific genes could be transferred experimentally to the Ti plasmid, and thence to plants, and the race was on to discover how to achieve this. By modifying the plasmid to remove the tumor-inducing genes, and adding the desired test gene with a suitable promoter to drive expression of the added gene, it proved possible to use modified *A. tumefaciens* Ti plasmids as vector, to transfer any desired piece of DNA from *Agrobacterium* to a plant cell.

The first widely accepted demonstration of successful transfer of exogenous plant DNA was the production of transgenic tobacco plants in 1983 using a modified *Agrobacterium* plasmid DNA[23,65–67]. Tobacco was often chosen for DNA transfer experiments because it was extremely amenable to plant regeneration from tissue and cell culture. One feature of the original approach was the use of a marker gene introduced alongside the transferred gene in order to select the transformed plants. Initially a gene for antibiotic resistance was used as a selectable marker but other markers were introduced later. The potential presence of antibiotic resistance genes in

plants was a matter of some concern for opponents of GM crops, but nowadays there are methods for removing these genes before the GM crops are used commercially.

The interaction of *Agrobacterium* with plants during the DNA transfer process involves complex molecular recognition and signaling, beginning with the production of acetosyringone by wounded plant cells, leading to the interaction between the bacterium and the plant cell, the excision of the T-DNA, and transfer to the plant, eventually culminating in the integration of the T-DNA into one or more of the plant chromosomes. This natural transfer of DNA between a bacterium and a plant, members of different biological kingdoms, evolved through a modification of the natural genetic and biochemical mechanisms involved in the transfer of DNA between bacteria. At first it was thought that *Agrobacterium* was only capable of transferring T-DNA to dicotyledonous plants, and could not be used to transfer genes to cereals, which are major food crops. As our understanding of the conditions required for DNA transfer to take place improved, however, this barrier was removed and Ti plasmids have been used to transfer DNA to several types of cereal crops.

Not surprisingly, many genes on the Ti plasmid are involved in the recognition and DNA transfer process, and the Ti plasmid is quite large. This led to the design of a binary vector system, consisting of two plasmids, one with many of the genes required for DNA transfer, and a second smaller plasmid carrying the T-DNA, into which the desired genes for transfer to the plant were inserted. This much smaller plasmid could replicate efficiently both in *Agrobacterium* and laboratory strains of *E. coli*, making it much easier to manipulate and assemble gene cassettes in the T-DNA for subsequent transfer to plants. A highly successful binary vector system was BIN19, developed by Bevan[68] and this was frequently used for early transformation experiments with *Agrobacterium*. A wider range of vectors is now available and their use has been reviewed by Lee and Gelvin[69].

As experience with *Agrobacterium* increased, simple methods were developed for its use in plant transformation, including introduction of the bacteria into plant tissues by vacuum infiltration and using a syringe barrel without a needle, which was particularly effective for leaves. Perhaps the most common procedure for transformation of the model plant *Arabidopsis thaliana* is now the “floral dip” method, where flowers are immersed in a suspension of *Agrobacterium*[70], and similar methods have now been developed for grasses such as *Setaria viridis*[71] and the oil crop *Camelina sativa*[72].

Initially, knowledge of the molecular cut-and-paste mechanism responsible for DNA integration was rather scanty. Insertion of added genes occurred randomly, and could even cause mutations by disrupting or influencing an existing gene. The extent to which this is a problem depends upon the relative density of functional and apparently neutral sequences in the genome of the target species and the ease with which transgenic plants can be generated. If the target species is relatively easy to transform, then it may be a simple matter to screen sufficient transgenic plants to identify one in which the transgene is expressed in an appropriate way and where the insertion into the genome has not taken place at a site that disrupts endogenous gene function. Bacterial or phage recombinase proteins recognize specific DNA sequences and can promote recombination between them. For example, the Cre recombinase will instigate recombination between two *loxP* sites. By including a *loxP* recognition sequences within a T-DNA, it is possible subsequently to insert additional DNA sequences into the first integration site if the new sequences carrying a matching *loxP* and a Cre recombinase is expressed at the same time as the new DNA is introduced[73]. It has been suggested that such an approach could be used for establishing sites, known as safe harbors where new transgenes could be safely integrated. However, such an approach is hampered by the fact that the initial integration events have to be screened and characterized to identify the safe harbor lines for subsequent use. Perhaps a more important use of recombinase systems is to flank the selectable marker on both sides with recombination sites so that it can be removed after transgenic plant regeneration following the transient expression of recombinase[74].

Targeted insertion through homologous recombination has been achieved in rice using T-DNA vectors in which a selectable marker (hygromycin resistance) was placed between two sequences homologous to the target locus and a gene conferring negative-selection (diphtheria toxin) was placed outside of the targeting sequences[75]. Plants in which the T-DNA inserted randomly receive both the hygromycin resistance and the diphtheria toxin genes, but where insertion is through homologous integration, the negative-selection sequences are excluded – allowing plant regeneration. However, even with this system, the recovery of homologous insertion events is rather inefficient. The efficiency of homologous recombination increases dramatically if a double strand break is first made at the integration target site[76,77].

As discussed later, it is now possible to add, change, or remove one or more genes in a nuclear or chloroplast chromosome, leaving behind no marker gene or other trace of intervention, apart from the intended gene(s).

4 First generation genetically modified crops—single gene resistances

The first generation GM crops were based on insertion of single genes from bacteria or viruses to confer new agronomic traits, such as resistance to herbicides, insects, and viruses (Table 1) or the inhibition of existing genes by sense or antisense gene silencing techniques (see Section 6), employing a modified *Agrobacterium* Ti plasmid

system as a vector. The CaMV 35S promoter was often used to control expression of these genes, with an additional selectable marker encoding antibiotic resistance in order to aid plant selection.

4.1 Herbicide resistance

Monsanto introduced crops resistant to glyphosate, a broad-spectrum herbicide (sold commercially as Roundup™) that kills most green plants by targeting the enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), which is required for aromatic amino acid biosynthesis. Metazoans lack this amino acid biosynthetic pathway and so for them glyphosate is not toxic. Resistance was conferred using a gene from *Agrobacterium* sp. strain CP4 that encoded an EPSPS resistant to glyphosate and this was introduced into several crops. Glyphosate can bind to the CP4 EPSPS but not in an inhibitory conformation, and a single-site mutation in the active site (Ala-100-Gly) restored glyphosate sensitivity[78]. With the aromatic amino acid biosynthetic pathway located within plastids, the bacterial enzyme had to be given a short leader sequence to ensure that the protein was targeted to this cellular compartment. The generation of plants resistant to this broad-spectrum herbicide enables farmers to spray the crop with glyphosate to kill weeds, thus reducing the competition for water, light and nutrients. An added benefit is that removal of weed species by herbicide treatment, rather than ploughing, has allowed the implementation of low tillage systems, which protect soil structure and reduce erosion. Subsequently, a range of different herbicide-resistance gene systems have been developed for other herbicides, using similar strategies.

As with all plant breeding, resistance can develop, but glyphosate-resistant superweeds can easily be killed by one of several other herbicides which have a different mechanism of action. Glyphosate has been widely considered to be nontoxic to humans, but in 2015 the International Agency for Research on Cancer, in Monograph 112 on glyphosate, concluded there was some evidence for it to be considered as a carcinogen. More recently, however, a review prepared for the New Zealand Environment Protection Authority concluded "…that – based on a weight of evidence approach, taking into account the quality and reliability of the available data – glyphosate is unlikely to be genotoxic or carcinogenic to humans and does not require classification under HSNO as a carcinogen or mutagen"[79].

4.2 Insect resistance

There are several naturally-occurring plant proteins, such as lectins and protease inhibitors, that interfere with insect feeding and these have been investigated for use as insecticides in GM crops. Highly effective insect resistance was achieved using a gene from the bacterium *Bacillus thuringiensis* encoding a Bt toxin that occurs naturally and kills insects that consume it. This bacterium has been used by organic farmers as a natural insecticide. Several companies, including Monsanto, isolated the gene, modified the codon usage so it was translated efficiently in plants, and expressed it in several crop species[80]. Several different types of Bt toxin exist which specifically kill caterpillars (Lepidoptera), fly larvae (Diptera) or beetles (Coleoptera)[81], allowing transgenic crops to be generated that are resistant to specific pests. The advantage of this is that it reduces the need for chemical insecticide sprays, only insects that eat the crop are affected, and it is effective at targeting insects buried in the plant body, which are often unaffected by chemical insecticide sprays. Transferring the gene for Bt toxin to plants could be regarded as preferable to spraying the whole bacterium expressing thousands of genes. Only insects that eat the crop are harmed and not those on nearby plant species. Again, there is a chance that resistance may develop, but it has been suggested that this possibility would be greatly reduced by pyramiding several Bt genes encoding proteins with different sequences[82,83].

4.3 Virus resistance

Virus resistance genes can be found in relatives of crop plants and introduced by conventional breeding, but examples of this are relatively rare. Research into the phenomenon of cross protection, where inoculation of a plant with a mild strain of a virus protected it against a subsequent infection by a related but more severe strain led to the discovery that expressing a virus coat-protein gene in plants could achieve a similar level of cross protection. This was first achieved for tobacco mosaic virus in transgenic tobacco, but has been demonstrated for many different viruses and crop species and has been used successfully to breed virus resistant GM crops. Some of the early virus resistant crops commercialized include squash and zucchini resistant to zucchini yellow mosaic virus and watermelon mottle virus 2, NewLeaf™ Y potato resistant to potato leaf roll virus and potato virus Y, plum resistant to pot virus, and papaya resistant to papaya ring spot virus[84–87]. In some cases, resistance may result from production of coat-protein fragments at altered stoichiometry that disrupt normal virus particle assembly. However, in most cases it was found that the level of resistance was highest in plant lines that only made small amounts of the transgenic coat protein. This led to the discovery that the main mechanism of resistance was based not on protein but on the production of a (double stranded) RNA homologous to the invading virus[88], and the mechanism seems to involve the destruction of the virus RNA by the siRNAs involved in gene silencing. Gene silencing is discussed further in Section 6. General strategies for production of virus-resistant crops have been discussed by Sudarshana et al.[89].

5 Engineering multiple traits through gene stacking in plants

The rapid advances in genome sequencing, bioinformatics and understanding of metabolic pathways have led to more and more candidate genes becoming available for trait modification or enhancement. Consequently, the focus is shifting from introducing traditional single traits, such as herbicide tolerance or insect resistance, to combinations of multiple traits or complicated metabolic pathway engineering in plants, especially main crop species. Modern societies demand not just enough food to feed the increasing population but also crops with enhanced nutritional value and improved tolerance to biotic and abiotic stresses to ensure a high productivity, with lower inputs of chemicals and water, even under unfavorable climatic conditions. As pollution and deterioration of the environment are becoming of greater concern worldwide, there is a high demand to replace fossil fuel with sustainable plant sources of materials and energy. Progress has been made in using plants as the source of cellulose for multiple purposes including cellulosic ethanol production and as a way to produce materials such as plastics, biodiesel, or even pharmaceutical components. These are all a reflection of the shift from first generation input trait to second generation output trait products[90]. The first generation products, such as herbicide tolerant GM crops, were often perceived to benefit only companies and farmers, and the second or future generation of products should bring nutritional, environmental and other benefits that consumers can directly enjoy[90]. Such products would have a much better appeal for consumers and would bring significant benefits to society. The statistics are showing a trend in this direction. In 2003, 8% of the commercially grown GM crops contained two or more traits[91], and this increased to 28% in 2014[92].

A key challenge, however, impeding the development of GM plants with multiple traits, or altered complex pathways, is the delivery of a large number of target genes into a plant genome. The conventional approach to stack genes in a single cassette is limited by the construction challenges, including availability of restriction sites, choice of promoters, the large size of the T-DNA, and potential instability, but inexpensive DNA synthesis and new cloning techniques such as Golden Gate and Gibson assembly and other promising technologies have been deployed to accelerate the engineering of multiple traits[93].

5.1 Repeated recombinase-mediated DNA cassette exchanges

Recombinase-mediated cassette exchange (RMCE) technology has been developed as a way to deliver a set of genes in a cassette to a specific site in the genome that is generated and selected from previous transformations[73,94], and this technology has been developed as a method to stack many more genes at the same genomic locus. Building upon the success in developing an FLP-FRT (flippase-flippase recognition target) RMCE technology in soybean[95], Li and his team developed this technology to stack seven trait genes at one genomic locus[96]. The system used two rounds of site-specific integration (SSI) of DNA to stack the seven genes. In the first round of SSI transformation, a gene silencing cassette, designed to simultaneously silence the soybean genes for fatty acid v-6 desaturase 2 and acyl-acyl carrier protein thioesterase 2 to improve oleic acid content, was inserted by RMCE at a pre-selected and characterized genomic site through biolistics of embryogenic cultures. In this construct, a new flippase recognition target (FRT) fragment (FRT12) was embedded behind the second selectable marker gene hygromycin phosphotransferase (*HPT*). In the subsequent round of transformation, selected transgenic plants were retransformed with the second DNA containing a diacylglycerol acyltransferase gene from *Yarrowia lipolytica* to increase oil content by the enhancement of triacylglycerol biosynthesis and three other genes, a *Corynebacterium glutamicum* dihydrodipicolinate synthetase gene, a barley high-lysine protein gene, and a truncated soybean cysteine synthase gene, to improve the contents of the essential amino acids lysine and methionine.

This system has several advantages. It overcomes the challenges of stacking many genes in a single construct due to limited cloning sites. Two groups of genes on different plasmids can be used, and this has the significant advantages of flexibility and feasibility for the cloning. In addition, the process of cassette exchange prevents complicated or fragmented insertions, thus generating a high frequency of clean insertions, which overcomes a major limitation for successful generation of transformants. Another major advantage is that the genes remain tightly linked and cosegregate through generations, ensuring that all the components of multiple traits or parts required for a complicated metabolic pathway remain present and intact. In cases where two selectable markers are used in the system, eventually only one selectable marker remains in the genome because the selectable marker inserted in the first round of cassette exchange, such as the HPT marker gene, would be excised in the second round of cassette exchange.

Unfortunately, not all gene insertion sites are equivalent and it is necessary to screen the sites to ensure that they meet the requirements for regulatory approval, including lack of disruption of other genes either by the physical disruption of a viable coding sequence or by affecting the expression of adjacent functional genes caused by the promoter of the trait genes. In addition, the transformation efficiency for these sites also needs to be assessed before

they can be used as a reliable vehicle for product development.

5.2 Co-transformation

Co-transformation has been used extensively in gene testing and product development in various species. Both direct (such as biolistics) and indirect (such as *Agrobacterium*-mediated) co-transformation methods have been used. Early co-transformation studies showed the feasibility of stacking genes in one transformation without the need to put all genes on a single construct. Depicker et al.[97] demonstrated that the frequency of co-transformation of two T-DNAs equalled the product of the individual T-DNA transformation frequencies when tobacco protoplasts were co-infected with a mixture of two *Agrobacterium* strains each carrying one Ti plasmid. Many subsequent studies in *Brassica* or tobacco showed a high frequency of linkage of the two T-DNAs[98,99]. With the progression of transformation technologies, co-transformation has been used to study complex metabolic pathways. For example, when studying the lignin biosynthesis pathway, Li et al.[100] infected tobacco with a mixture of different *Agrobacterium* C58 strains carrying four genes independently and obtained transgenic plants with co-transformation of one, two, three and four T-DNAs at a frequency of 35%, 27%, 19% and 19% respectively[100]. This strategy helped the team to study the key enzymes in the lignin biosynthesis pathway in a very efficient way. The same strategy was applied to manipulating the key enzymes of the lignin biosynthesis pathway in aspen[100]. By co-transformation of aspen with one construct overexpressing ferulate 5-hydroxylase gene and another downregulating the 4-coumarate-CoA ligase gene, transformed plants with an increased ratio of syringyl lignin to guaiacyl lignin and lower overall lignin content were obtained. Such trees are much easier to pulp, and thus the chemical and energy use required for pulping and paper-making are significantly reduced. An unexpected benefit was that the cellulose content was increased while reducing the lignin[101]. Similarly, a co-transformation strategy was used to engineer the carotenoid biosynthesis pathway in important crops such as rice and maize. In rice, both *Agrobacterium*-mediated and biolistics-mediated co-transformation methods were used successfully to engineer the β-carotene (provitamin A) biosynthesis pathway to be expressed in rice endosperm[102,103]. When *Agrobacterium*-mediated co-transformation was conducted, *Agrobacterium* strain LBA4404 containing either plasmid pZPsC or pZLeyH were mixed to infect rice immature embryos[102]. pZPsC contained a daffodil *psy* gene coding for phytoene synthase and a bacterial *crtI* gene coding for phytoene desaturase. The plasmid, pZLeyH, contained a daffodil *lcy* gene coding for lycopene β-cyclase and hygromycin resistant selectable marker gene *aph IV*. Ten of the 60 lines recovered had all four genes, and one line had an accumulation of 1.6 $\mu g \cdot g^{-1}$ carotenoid in the endosperm. The level was close to the recommended daily intake target of 2 $\mu g \cdot g^{-1}$ carotenoid. Similarly, the Golden Rice phenotype was introduced to seven commercial cultivars of indica and japonica rice through biolistics-mediated co-transformation, and the β-carotene level in T1 seeds of one transgenic line of Nang Hong Cho Dao NHCD3 reached 1.05 $\mu g \cdot g^{-1}$[103]. These studies paved the way for the development of improved Golden Rice 2, in which the daffodil *psy* gene was replaced with one from maize. These plants accumulated 37 $\mu g \cdot g^{-1}$ carotenoid in rice grains[104]. In an attempt to understand the complexity of the carotenoid biosynthesis pathway, multiple genes were co-transformed into a white maize cultivar using biolistics[105]. In the study six genes including five carotenogenic genes: *Zmpsy1* (Zea mays phytoene synthase 1), *PacrtI* (Pantoea ananatis phytoene desaturase), *Gllycb* (Gentiana lutea lycopene cyclase), *Glbch* (Gentiana lutea carotene hydroxylase, a plant-type-ring nonheme di-iron monooxygenase introducing hydroxy groups at C-3), and *ParacrtW* (Paracoccus carotene ketolase) and the selectable (herbicide resistance) marker *bar* gene were coated on gold particles and used to bombard maize immature embryo. Transformants with various combinations of the carotenogenic genes were generated and showed a range of levels of carotene and other carotenoids. The success of these studies revealed the potential of co-transformation to modify metabolic pathways efficiently and studies on fortifying several other crops, including banana, are underway. Plants modified in this way have potentially significant nutritional and medical implications, but this has been opposed, particularly by Greenpeace. In 2016, in a letter published by the Washington Post, 167 Nobel Laureates said: “We urge Greenpeace and its supporters to reexamine the experience of farmers and consumers worldwide with crops and foods improved through biotechnology, recognize the findings of authoritative scientific bodies and regulatory agencies, and abandon their campaign against GMOs (genetically modified organisms) in general and Golden Rice in particular”[106].

The ability to modify multiple genes in complex metabolic pathway through a single transformation process is very powerful. For plant species such as trees, which usually take a longer time to go through the regeneration cycle, co-transformation offers the opportunity to study multiple genes in one transformation event and can significantly accelerate the gene evaluation process. Co-transformation tends to produce unlinked genes which would be useful to remove undesired components such as selectable marker gene by subsequent breeding. For product development, simple, clean and non-disruptive insertions are needed to produce transformants for the regulatory process, but this can be very burdensome, as the co-transformation tends to yield events with complicated insertion patterns and segregation may not follow a simple Mendelian pattern, at least at the T1 level[102].

5.3 New technologies for stacking genes

5.3.1 Polycistronic gene expression cassettes

Recent development in chloroplast transformation have shown great potential for stacking genes. Chloroplast genomes retain key prokaryotic features such as gene arrangement in operons and transcription of polycistronic mRNAs. It would be a lot simpler and easier for multiple gene manipulation if genes could be stacked in operons and driven by a single promoter with multiple coding sequences in tandem. However, the translation of polycistronic transcripts is not as simple in chloroplasts as in bacteria. In bacteria, polycistronic transcripts get translated directly, but in most cases in plant chloroplasts they are cleaved into stable monocistronic or oligocistronic transcripts then recognized and translated[107]. While operons psbE, psaA/B and petA are transcribed without further processing, transcripts of other chloroplast operons have to undergo RNA cleavage (also called intercistronic processing) to be translated as monocistronic or oligocistronic transcripts[108]. Failure of translation of polycistronic transcripts is considered to be the main problem causing low or no gene expression[109,110], but intercistronic processing would reduce the risk of poor gene expression for genes in an operon in the chloroplast genome and increase the predictability of the expression of these genes[108]. With the hypothesis that certain sequences facilitate consistent and efficient cleavage of the psbH RNA from the polycistronic transcripts of the psbB operon, Zhou et al.[108] mapped the intercistronic cleavage sites upstream and downstream of psbH and identified an intercistronic expression element that mediates efficient intercistronic cleavage of polycistronic mRNAs into stable monocistronic transcripts. This success paved the way to engineer the vitamin E biosynthesis pathway in transgenic tobacco and tomato plastids with a synthetic operon with cyanobacterial genes coding for homogentisate phytyltransferase and tocopherol cyclase, and an *Arabidopsis* gene coding for γ-tocopherol methyltransferase leading to accumulation of tocochromanols (tocopherols and tocotrienols)[111]. In comparison to the wild type, the transgenic tobacco or tomato lines had up to a 10-fold higher accumulation of tocopherol in leaves and a threefold increase in green tomato cultivars Dorthy's Green and Green Pineapple. The accumulation of tocopherol also improved the cold tolerance of the transgenic plants. This technology provides a powerful tool to design artificial operons and use chloroplasts to express multiple genes from an operon in a coordinated pattern. The success in engineering the vitamin E pathway in tomato also showed the feasibility and potential to improve the nutritional value of important food crops. The key question is how to make this a versatile tool for many plant species especially the major crop species. Unlike nuclear transformation, *Agrobacterium* cannot be used for delivering DNA to the plastid and instead biolistic approaches are used. In addition, given that there are often 100 chloroplasts in a leaf cell, each with about 100 copies of the chloroplast genome, it is important to maintain selection until all wild type genomes have been replaced with the transgenic ones. If this is not done, the chloroplasts containing the transgene can be displaced and lost. At the moment, transformation of chloroplasts is difficult for many plants especially monocot crop species, and it is unclear if there is a size or number limitation on the number of genes of interest that can be included in the operon to be inserted into the plastids. In addition, more work is needed on controlling the developmental timing and expression level of the genes of interest. Another concern is the ability to titrate the level of expression. While conventional transformations can use promoters of various strength levels to control the expression of the gene(s) of interest, expression of the operons in the plastid genome is much higher because of the sheer number of plastids and genome copies per plastid in a cell, relative to nuclear genes. The limitations of polycistronic gene expression in chloroplasts could be overcome by employing a 2A self-cleaving peptide system. Picornaviruses, for example foot-and-mouth disease virus, use 2A peptides to mediate cleavage between two proteins by a ribosomal skip mechanism[112].Taking advantage of this, different genes linked by a 2A peptide coding sequence could be co-expressed from a single open reading frame[113] and subsequently cleaved, liberating the separate enzymes.

5.3.2 Compact trait loci generation through site-specific integration

When dealing with metabolic pathway engineering or stacking multiple traits, it is desirable to physically link introduced genes of the pathway(s) so that all components are more likely to be inherited together in the breeding process. Previously we discussed the use of repeated SSI transformation to stack seven genes at one site[96]. An even more powerful strategy is to create multiple loci that are close to each other on a chromosome so that the traits or genes in a metabolic pathway can be introduced individually and linked together. For SSI sites, there are several requirements to make this possible. First, a highly efficient transformation system to deliver the construct for target site creation is needed. A large number of transgenic events have to be created. Secondly, the insertion events should be clustered closely on the same chromosome. Thirdly, these clustered sites should be constructed bearing in mind the requirements in order to obtain regulatory approval. A significant effort is necessary to generate these sites, but their locations can be preselected by using a CRISPR-Cas (CRISPR: cluster regularly interspaced short palindromic; Cas: CRISPR-associated) system[114] (also see Section 7).

With CRISPR-Cas technology, the target loci can be preselected and used to generate the SSI sites. Multi-gene cassettes conferring desired traits can be delivered to these sites using SSI technology and become linked. Alternatively, they can be directly introduced to sites where double-stranded breaks are generated by the CRISPR-Cas system[115,116]. This also requires a highly efficient transformation system to deliver the construct.

6 Gene silencing and RNAi

6.1 Gene silencing: discovery and applications of antisense genes, posttranscriptional gene silencing and RNAi

In plant breeding it is sometimes necessary to confront the fact that natural processes do not always coincide with the needs of human consumers and it may be desirable to prevent the action of specific genes. Powerful strategies for switching genes off have been developed which are very effective in modifying plant gene expression by taking advantage of endogenous mechanisms that recognize and destroy antisense RNA. The first attempt at this was to use transient expression of the bacterial gene chloramphenicol acetyltransferase gene from plasmids in either the sense or antisense orientation in carrot protoplasts. When both constructs were expressed together, transcription of antisense RNA inhibited sense-gene mRNA accumulation[117]. Soon after, *Agrobacterium*-Ti plasmid-mediated transformation was used to stably integrate antisense transgenes into tomato and petunia plants to downregulate endogenous homologous genes. These experiments, which used the CaMV 35S promoter to drive expression of the antisense gene, were remarkably effective at knocking down, and in some cases almost eliminating, the accumulation of the mRNA from the target genes such as polygalacturonase (PG) in tomato fruit[118,119], and chalcone synthase in petunia flowers[120]. Antisense transgenes are stably inherited, but by selfing hemizygous PG antisense plants, some non-silenced progeny were recovered which retained a fully functional PG gene but lacked an antisense gene[121]. This indicated that the PG gene was not permanently disrupted but could be inhibited when both sense mRNA and antisense RNA from the antisense gene were present in the same cell (Fig. 2). Smith et al.[118] speculated that: "The low level of PG mRNA observed in ripe fruit… may point to some process occurring in the nucleus, interference with transcription, processing or transport, for example, rather than inhibition of translation by the formation of RNA/RNA hybrids in the

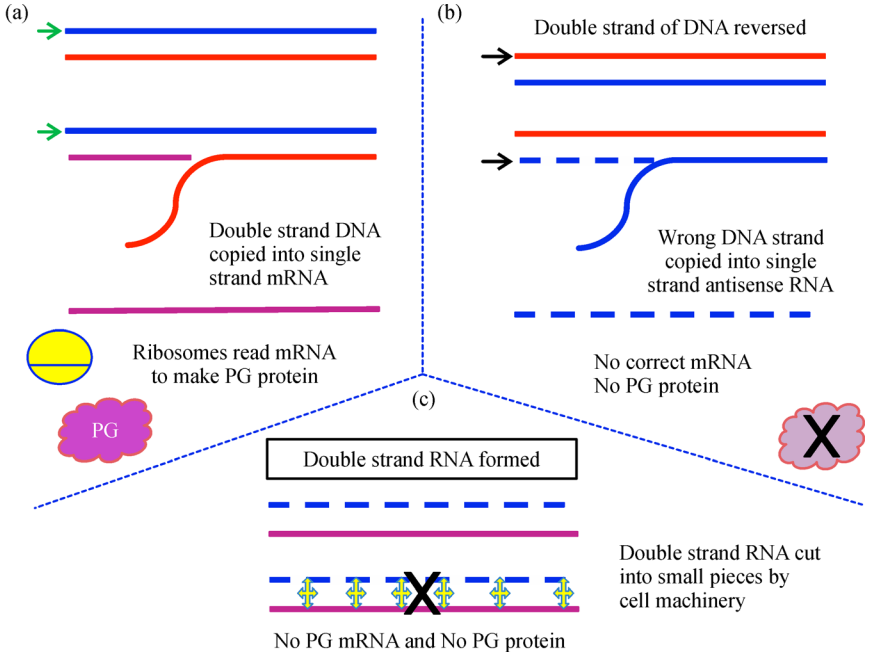

Fig. 2 Antisense RNA causes posttranscriptional mRNA degradation and effective inhibition of polygalacturonase gene expression. The diagram depicts three scenarios. (a) Synthesis and translation of polygalacturonase (PG) mRNA during ripening of tomato; (b) transcription of a PG coding sequence inverted with respect to the promoter, as happens in all cells of the PG-antisense GM tomatoes[118]. Similar results were obtained, in some transformation events, with a PG sense gene[121]; (c) the situation when the transcription of the PG gene is switched on by the ripening control system in the GM tomatoes. The sense and antisense RNA form a double-stranded hybrid which is recognized and degraded by nucleases. The mechanism probably involves the cell's siRNA system, targeting all related sequences for destruction.

cytoplasm. Alternatively, the low levels of PG mRNA could be caused by the selective degradation of double-stranded RNA hybrids."

Notably, both antisense and sense (as control for the antisense) constructs driven by the CaMV 35S promoter showed a silencing effect[122–124], a phenomenon that came to be known as co-suppression or posttranscriptional gene silencing (PTGS). Various mechanisms were proposed to explain PTGS, including the suggestions it was linked to abnormal RNA arising from a transgene or virus, or very high levels of RNA due to strong expression from the transgene(s) or multiple insertions of the transgene, or the production of aberrant RNA or transcription from repeated DNA inserts. The highly effective silencing of genes with both sense and antisense constructs varied when the same gene constructs were added in different transformation events and located at different insertion sites, indicating that where or how the gene was inserted may be important. The strong similarity in the silencing effect caused by both sense and antisense transgenes prompted some researchers to hypothesize that they may share the same mechanism[125].

One early hypothesis was that, since most transgenes are constructed from cDNAs, their mRNAs might be processed differently from those mRNAs transcribed from authentic endogenous genes, and hence they could be sensed as aberrant. It was also proposed that transgene repeats may be sensed as invading DNA (or their RNA transcripts) and trigger the silencing mechanism. A transgene with direct repeats from a virus produced more plants with resistance to the virus[126]. Virus RNA might also be sensed as abnormal, and could be used as templates by RNA-dependent RNA polymerases (RdRPs) to generate double-stranded RNA (dsRNA) and initiate silencing. This theory is supported by links between RNA silencing and mRNA turnover or mRNA processing[127–129].

Another hypothesis was that introduction of transgene(s) causes the combined level of mRNA from the transgene and homologous endogenous transcripts to rise above a certain threshold, triggering the initiation of gene silencing. Insertion of multiple transgenes into the plant genome can occur during transformation[130] and transgene duplication could cause epigenetic changes, leading to altered efficiencies of mRNA maturation and export from the nucleus; this might provide mRNA templates for RdRP[131]. Multiple insertion of the transgenes could also lead to accumulation of very high levels of their transcripts, than from the homologous endogenous genes, and this might be enhanced by the use of the strong constitutive CaMV 35S promoter in much of the earlier transformation work. A high level of certain transcripts might be sensed by the cell as abnormal, which may affect their processing and initiate PTGS to reduce the level of the transcripts. The association between high level of transcripts and silencing was confirmed by the observation that a weaker promoter would reduce the frequency of silencing and high endogenous target transcript levels were required for silencing[132,133].

Later, virus-induced gene silencing (VIGS) reported by Baulcombe's group proved that RNA is the target and initiator of PTGS[134], and the technique has since been used for functional analysis of plant genes[135], in both model plants and economically important crops[136,137].

Hamilton et al.[138] discovered that a transgene with an inverted repeat caused a strong and high frequency of PTGS in tomato. Transcription of the repeat would be expected to produce a partially-double-stranded region in the transcript. A key discovery was the observation that plant PTGS is associated with the production of small antisense RNAs[139]. The mechanism of PTGS in plants was clarified by the discovery of the phenomenon of RNA interference (RNAi) in *Caenorhabitis elegans* by Fire et al.[140] where double stranded RNA causes silencing. These workers were awarded the Nobel Prize for Physiology or Medicine in 2006 for their discovery, and transformation constructs that produce double-stranded transcripts have become the method of choice for introducing dsRNA for gene silencing in plants. In brief, the mechanism of silencing involves dsRNA derived from a transgene containing inverted repeats, or by transcription of complementary RNA from the transgene mRNA by RdRP, which is then cleaved by an RNase III-like protein called Dicer into short RNAs (sRNAs) of 21–28 nucleotide. One of the strands acts as guide RNA and is integrated into an RNA-induced silencing complex containing an Argonaute protein, and the complex degrades the target RNA based on duplex formed between the sRNA and target mRNA[141].

Various constructs have been tested to find efficient and convenient RNAi transgene constructs for use in plants. Waterhouse's group showed that 98 to 853 bp hairpin RNA constructs can cause efficient silencing in different plant species, and inclusion of an intron as a spacer between the inverted repeats can improve the efficiency to 90%–100% of transgenic lines showing silencing[142]. Several vector systems have been developed to make constructs containing inverted repeats for expressing dsRNA in transgenic plants, such as ChromDB, Hellsgate, pHANNIBAL, pX7-RNAi and alcR-RNAi, and these have been reviewed by Yin et al.[143]

6.2 The potential of RNAi technology for plant improvement

The Calgene company used an antisense PG gene to produce their Flavr Savr tomato. The idea was to inhibit the expression of the cell-wall modifying gene encoding PG that was expressed during ripening and cause the fruit to soften more slowly so they could be left for longer on the plant to develop a better flavor. This was sold in the USA from 1994 to 1997 but was not a commercial success,

however, and production was discontinued. The Zeneca company, in conjunction with Nottingham University, targeted the same PG gene in tomatoes, using a sense-gene silencing approach to develop GM tomato puree which reduced waste and had improved viscosity[121]. This product was the first GM food to be approved for sale in the UK and USA and was sold commercially in UK supermarkets in 1996[144]. Initially it was produced in the USA and sold well in the UK, but faced regulatory delays and also faced opposition from some consumer groups and NGOs (nongovernment organizations) and its production was discontinued. Following these developments several other potential tomato GM products were developed but never marketed, including the discovery of ACC oxidase genes and the inhibition of ethylene synthesis[145].

Using antisense RNA/RNAi, any unwanted traits in plants could be potentially modified if the key genes related to the traits are known and if a transformation system has been established for the plant species in question. There are numerous research papers on traits altered by RNAi, which could be put into practice for higher yield, improved production and quality. Ripening-related genes such as ACC oxidases and ACC synthases can be silenced to extend the shelf life in tomato and other climacteric fruits[145,146]. Also, several studies have shown that RNAi can be applied to protect crops against plant pathogens such as viruses[147], bacteria and fungi[148] and possibly also some insect species[149]. RNAi-mediated male sterility could also be achieved by silencing genes that are essential for pollen development[150,151].

In the biofuel research area, RNAi-mediated silencing of key genes related to lignin biosynthesis in poplar, switchgrass, sugarcane and maize decreases lignin content, laying the foundation for improving poplar for biofuel[152–155]. Suppression by RNAi of glycosyltransferases essential for biosynthesis of glucuronoxylan during secondary wall thickening increases wood digestibility[156,157]. RNAi has also been used to manipulate oil content in seeds for biofuel production. Downregulation of a triacylglycerol lipase by RNAi in *Jatropha curcas* results in up to 30% higher total seed storage lipid[158]. Silencing of a multifunctional lipase/phospholipase/acyltransferase with antisense and RNAi enhanced lipid levels significantly without affecting growth in the microalga, *Thalassiosira pseudonana*, improving the economic feasibility of using microalgae[159].

RNAi technology has also been used to combat pests by generating transgenic plants expressing dsRNA that can act like species-specific insecticides, targeting essential mRNAs[160,161]. These dsRNAs are more effective, and work over a longer period to suppress target genes, compared to siRNAs[162]. If the dsRNAs are expressed in plants using a transgene that target insects, however, they will be processed into siRNAs by the plant silencing mechanism soon after they are transcribed, resulting in only a very low level of dsRNAs. This might be insufficient to kill the insects that consume the plant tissues, although it could cause some inhibition to the growth and development of the insects if crucial genes are targeted. A breakthrough was made recently, taking advantage of the lack of an RNAi mechanism in plastids, to generate transgenic potato plants that accumulated high levels of dsRNAs (as much as 0.4% of the total cellular RNA) in chloroplasts[163]. This dsRNAs was designed to target the β-actin gene of the Colorado potato beetle, and the transplastomic potato plants obtained are lethal to the pest larvae.

Another approach, called host-delivered RNA interference-triggered silencing of parasite-specific genes crucial for development, is also being explored for engineering resistance to parasitic plants[164]. Silencing of the mannose 6-phosphate reductase gene in the parasitic weed *Orobanche aegyptiaca*, achieved by expressing the homologous dsRNA sequences in the host tomato plant, led to a significant increase in the mortality of the parasite on the transgenic hosts[165]. Similarly, *Medicago truncatula* roots transformed with an RNAi construct targeting the cytosolic acetyl-CoA carboxylase gene from the parasitic weed, *Triphysaria versicolor*, reduced the *Triphysaria* root viability by up to 80%[166]. A similar strategy, however, applied in transgenic maize plants, using RNAi to target Striga genes, did not produce resistance against *Striga*, although the growth of the parasite was retarded to some extent in some lines[167]. A VIGS system has been developed for use in the parasitic plant *Striga hermonthica*, for identification of key parasite genes essential for development and parasitism, with a view to production of transgenic maize with resistance to the parasite using RNAi[168].

Using the model plant *Arabidopsis*, transgenic plants expressing dsRNA targeting genes related to parasitism or development of nematode parasites (mostly root-knot nematodes) show resistance, by suppression of parasitism, causing reduction in the number of developing female parasites and retardation of growth[169–172]. Similarly, transgenic tobacco lines expressing dsRNA targeting housekeeping genes of root-knot nematode showed silencing of the target genes and protected the plants from infection[173]. Recently, plant-mediated RNAi of two essential genes (heat-shock protein 90 and isocitrate lyase) resulted in transgenic *N. tabacum* plants resistant to the root-knot nematode, *Meloidogyne incognita*[174]. There is no potato cultivar with resistance to any *Meloidogyne* sp., resulting in serious problems for potato production. Dinh et al.[175] produced transgenic potato plants expressing dsRNA of the *Meloidogyne* 16D10 effector gene, which showed significant broad resistance to the five *Meloidogyne* sp. tested, including the most important species affecting potato. Thus, although still in the early stage, the application of RNAi in crop protection strategies against

nematodes offers a promising future[176], especially in situations where there is no naturally resistant germplasm available[148].

6.3 RNAi-based genetically modified plants in the field and marketplace

The first crops marketed using RNA-based silencing techniques were the Flavr Savr tomato and the low PG tomato puree described above (Section 6.2). By 13 January 2016, 121 GMOs covering 20 plant species and many traits had been listed by the US Department of Agriculture (USDA) as petitioning for determination of nonregulated status. Many of these traits involve modifications with RNA silencing techniques (USDA, https://www.aphis.usda.gov/biotechnology/petitions_table_pending.shtml).

Resistance to viruses has been achieved by RNA silencing of the viral genes in squash (by The Upjohn Company), papaya[85] and plum[87]. Originally, virus resistance was believed to be caused by overexpressing coat protein but, as described above, it is now clear it involves an RNA silencing mechanism. RNAi has also been used successfully for the development of transgenic cassava resistant to cassava brown streak virus and cassava mosaic virus, plum resistant to plum pox virus, potato resistant to potato virus Y, and transgenic potato and wheat resistant to fungal pathogens[84].

The larval stages of some insect pests have been successfully targeted by the expression of dsRNA corresponding to essential insect genes in plant tissues that are consumed by the larvae. For some insect species, such as the western corn rootworm, ingestion and uptake into the gut of such RNAs is sufficient to trigger gene silencing and insect death[149].

Transgenic soybean cv. Plenish, produced by Dupont/Pioneer, with high oleic acid, and cv. Vistive Gold, produced by Monsanto, with low saturated fats, are already marketed in the USA. Several potential GM plants that use RNAi technology such as camelina with long chain omega 3 polyunsaturated fatty acid, gluten-free or high amylose wheat, and drought tolerant corn, are at the research and development or field evaluation stages and have been reviewed by Ricroch and Hénard-Damave[84].

In 2015, the USDA approved[177] marketing of browning resistant GM potatoes produced by the US company JR Simplot of Boise, and nonbrowning Arctic Apples produced by the Canadian firm Okanagan Specialty Fruits[178]. Both products were developed by down-regulating polyphenol oxidase (*PPO*) genes based on the RNA silencing mechanism, with slight differences. In the GM apples, antisense RNA is transcribed from an antisense PPO transgene, which forms dsRNA with endogenous PPO mRNA and initiates silencing, whereas in the GM potato, inverted repeats of a PPO gene fragment are transcribed, generating siRNAs to target endogenous PPO mRNA degradation. A GM potato has also been modified by RNAi to silence the asparagine synthetase-1 gene for reduced acrylamide production upon cooking[177].

7 CRISPR/Cas9-defense mechanism against invading DNA and RNA heralds a technological revolution for genetic modification of plants

Bacteria contain systems for detecting and destroying invading DNA and various components of these systems have been used as tools in molecular genetics for cloning, targeted gene editing and replacement in directed breeding of microbes, animals and plants. Restriction endonucleases were the first proteins discovered to cleave DNA at specific points[179] and made it possible to manipulate gene sequences. Later, some zinc finger proteins, such as mouse Zif268 (or EGR1), were found to be able to bind distinct DNA triplet recognition motifs, and were modified by fusing with FokI nuclease to form zinc finger nucleases (ZFNs) capable of recognizing a specific site, for manipulating a target gene in a genome for functional analysis, therapeutic application and crop improvement[180,181]. Soon, a similar strategy using Transcription Activator-Like Effectors fused with Nucleases (TALENs) was developed and utilized for genome editing including the production of disease resistant rice and wheat[116,182]. For each target site, however, a specific chimeric ZFN or TALEN protein is required to be engineered in order to recognize the site and this is a complex, expensive and time consuming process that greatly hinders their use.

Recently, however, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) / CRISPR-associated protein (Cas9) system has become available and has, in a very short time, become the method of choice for targeted genetic modification of genomes from microbes, animals and plants. CRISPR/Cas9 is a bacterial type II adaptive immune system, which consists of three components, Cas9 protein, CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA)[114,183–185]. Sequences of invading DNA are processed and stored as 20 nucleotide fragments in the bacterial CRISPR array region to provide an archive of sequences from past infections. Recently, it has been discovered that some CRISPR systems can also store RNA sequences, through the action of a natural reverse transcriptase-Cas1 fusion protein[186]. This array, containing the stored DNA fragments, is transcribed and processed to form crRNAs, which associate with the tracrRNA and the Cas9 protein. If the bacterium is invaded by the same foreign DNA, such as a virus, the crRNA, which contain a 20-nt sequence homologous to the invading DNA, will target Cas9 nuclease to the invading sequence which is then cleaved. The tracrRNA stabilizes the complex and activates the Cas9 nuclease to cleave the DNA, generating double-strand breaks[114,187]. The system has been modified into a two-, rather than a three-

component system by combining the crRNA and tracrRNA as a single guide RNA (sgRNA) for the convenience of application in genome editing[183].

There has been rapid and widespread application of the CRISPR/Cas9 system for genome editing in animals and plants, for analysis of gene function, breeding organisms with new traits, and for investigations of therapeutic potential[187–189]. Early investigations to test the use of CRISPR/Cas9 system in plants showed positive results in protoplasts and calli, and also by agro-infiltration of leaves[190–192]. Now, a modified CRISPR/Cas9 system, consisting of a Cas9 codon-optimized for plants in a cassette driven by a plant ubiquitin promoter with sgRNA controlled by plant U6 promoters, is available and has been used to generate targeted and stable mutations in *Arabidopsis*, tomato, rice and maize[193–196].

CRISPR/Cas9 is highly efficient, with a high degree of target specificity, but examples of off-target events have been reported, mostly in animal systems[197] but also in plants[198]. It is clear that sgRNAs of different designs and Cas9 orthologs from different species are involved in differences in specificity of the CRISPR/Cas9 system[197]. Efforts are being made to enhance the specificity by screening for Cas9 of higher specificity, modifying Cas9 and introducing a second sgRNA to cause single strand breaks on each of the double strands of the target site[197,198]. CRISPR/Cas9 systems for editing multiple genes, especially for manipulating a gene family, a pathway and multiple sites within a gene, have been developed[196,199–203].

By inactivating the nuclease activity of Cas9 via modification of the catalytic domain, a deactivated form of Cas9 (dCas9) can be fused to transcriptional activators, repressors and chromatin remodeling factors, to regulate target genes at the transcriptional level using the promoter sequences of the target genes in the sgRNAs[203].

The CRISPR system has also been applied for targeted gene integration in conjunction with homologous recombination e.g. insertion of a strong promoter upstream of a Myb transcription factor gene controlling anthocyanin biosynthesis in tomato[204] and integration of herbicide resistance genes in soybean and rice[205,206].

The CRISPR system has great potential for crop improvement[207–209] and has already been shown to improve disease resistance to powdery mildew in bread wheat[116], and has led to herbicide resistance in soybean and rice[206]. The system is highly versatile, with many applications, including inactivation or activation of genes by regulatory factors fused with dCas9, or modification of regulatory elements in a promoter and mutation of the coding regions, and integration of new genes or regulatory sequences such as promoters or other elements by homologous gene targeting. This raises the theoretical possibility of modifying any crop trait in a highly specific manner. By combining this technology with advances in synthetic biology and methods for constructing vectors containing multiple genes, we would expect to see a dramatic increase in the number of crop lines with a range of new and complex traits modified through genome editing with the CRISPR system.

It is important to emphasize that the CRISPR system also makes it possible to produce crops with transgene technology that are indistinguishable from traditionally generated crops. This can be achieved by carrying out all of the targeted modifications at the desired locus or loci, including use of synthetic designer DNA, and subsequently removing all traces of the transfer, integration, or selection process used. Outcrossing can also be used to remove any off-target events. Thus, endogenous genes could be targeted for mutations for improving certain traits by stable transformation with the CRISPR system, then the CRISPR transgene could be segregated out by selfing, and selecting T2 progenies for lines with the targeted gene modified but lacking any transgene used in the process[210,211]. This raises the critical issue in GMO regulation of: how should crops generated through such genome editing be regulated? According to the USDA, plants modified with genome editing methods such as the CRISPR/Cas9 system could be excluded from GMO regulations as they are free from transgenic sequences or pathogenic *Agrobacterium*; but might still conflict with EU regulations[211,212]. A regulatory framework for genome-edited crops (GECs) has been proposed in order to promote the application of genome editing technology in crop breeding and the acceptance by regulatory authorities and the public that GEC products are similar to those derived from cross-breeding[213]. The opportunities offered by advances in knowledge in synthetic biology, plant and crop biology, and technology for genetic modification make resolution of this issue an urgent international priority.

8 Future prospects

In an age where the production of food is threatened by the impact of climate change and population pressures, it is essential that we use all available science and technology to ensure food security for all. Now that we have the complete DNA sequence of many crop plants, these new gene technologies are revolutionizing our understanding of how plant genes work, how they control growth, yield, resistance, and nutritional properties, and can greatly increase the speed and precision of plant breeding processes. This makes it possible to produce new plant cultivars with desirable characteristics much faster, and achieve outcomes sometimes thought impossible with established breeding approaches. Synthetic biology offers the opportunity of developing new plants with major changes in their characteristics capable of overcoming a range of challenges. Genetic modification is almost

certainly not the only answer to the problems of global food production, but it can contribute an important part of the solution.

Plant-based oral vaccines may transform the vaccine industry, because they offer a number of advantages for producing large quantities of vaccines at low cost, using proteins expressed in the plant cytoplasm, or sometimes in significantly larger quantities, in the chloroplasts. They offer the prospect of being free from contamination with animal products, with implications for improved safety, ease of storage and distribution, and low cost of production. A number of subunit vaccines and monoclonal antibodies have been produced in plants and tested against a range of diseases[214–216]. This raises the future prospect of using plants as production systems and also as therapeutic fruits or vegetables with specific health protection benefits, perhaps alongside vitamin-enhanced fruits and vegetables.

With the advent of CRISPR, targeted gene modifications will be indistinguishable from a natural mutation. New cloning techniques, inexpensive DNA synthesis methods and advanced transformation technologies mean that it is possible to contemplate assembly and transfer of multi-gene pathways and regulatory circuits to GM plants. This will enable more rapid introduction of novel characteristics, producing new crop cultivars with improved nutritional value, requiring lower inputs, and capable of adapting to new environments. Despite the public concerns or opposition in some regions, the advantages of GM crops are well recognized and accepted by the majority of countries in the world. In a recent letter in Science, Fahlgren et al.[217] wrote, "Current use of genetic modification technology for crops is safe and effective, and future use should be guided by scientific evidence."

Acknowledgements This work was supported by the National Natural Science Foundation of China (31371693, 31471556, 31600289 and 31471502).

Compliance with ethics guidelines Xingchun Wang, Shujun Chang, Jie Lu, Rupert Fray, Don Grierson, and Yuanhuai Han declare that they have no conflict of interest or financial conflicts to disclose.

This article is a review and does not contain any studies with human or animal subjects performed by any of the authors.

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