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All-native DNA transformation: a new approach to plant genetic engineering

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Crop genetic engineering relies on the introduction of foreign DNA into plant genomes. Although genetically engineered traits provide valuable alternatives to those available through conventional breeding, there is public concern about the consumption of foods derived from transgenic plants. This concern raises the question of whether crops can be improved by inserting only native DNA into their genomes. Here, we discuss how rapid advances in molecular biology make it possible to use plants themselves as DNA sources. Native genes and regulatory elements can be reintroduced into plants without the need to use selectable markers. By also using transfer DNAs that are derived from within the targeted compatibility group, genetically engineered plants can now be produced that lack any foreign DNA.

Recombining the genetic material that is available within commonly available gene pools has traditionally increased productivity in most crops. Although the systematic creation of new varieties has supported a doubling of yields over the past 40 years, it is doubtful that the available diversity can sustain much further yield increase [1]. Efforts to exploit the vast genetic diversity found in exotic germ plasm has gained momentum with the development of DNA markers and comprehensive molecular maps, allowing the transfer of beneficial loci with minimal linkage drag. QUANTITATIVE TRAIT LOCI (see Glossary) for disease resistance, enhanced yield and other important traits are being introgressed from wild relatives and related species through wide crosses. SOMATIC CELL HYBRIDIZATION techniques made it possible for plant breeders to cross species boundaries [2] and to delve even deeper into the richness of exotic gene pools.

Eager to accelerate the process of crop improvement, plant biologists domesticated the cross-species DNA transfer system of the plant pathogenic bacterium *Agrobacterium tumefaciens* and developed the first transgenic plants containing fungal and bacterial DNA. During the subsequent two decades, a multitude of genetically modified plants were generated that contain DNA that could not have been introgressed through any available breeding method. An initial lack of knowledge about the molecular biology of plants limited the ready exploitation of these sources for crop improvement. As a result, genes for agronomically important traits such as herbicide tolerance and insect resistance were mainly derived from other organisms, primarily bacteria and viruses, whose genomes can be more easily subjected to molecular analysis. In addition to the foreign genes of interest, most transgenic plants contain bacterial selectable marker genes that provide tolerance of antibiotics, herbicides or drugs. Although the stable integration of such genes makes it possible to identify the rare transformed cells and to regenerate plants from them, their lingering presence in crops complicates the regulation process and negatively affects public acceptance of the final products [3].

The divide between conventional breeding and genetic engineering became more explicit with the creation of synthetic genes. Instead of using *Bacillus thuringiensis* (*Bt*) insecticidal genes for insect control, synthetic variants were designed whose codon usage bias resembled that found in plants [4]. Compared with the original bacterial *Bt* genes, expression of the synthetic derivatives provided higher levels of insect tolerance in transgenic plants. Test tube evolution was further exploited by shuffling homologous genes, thus creating libraries of chimeras that can be screened for optimal activity [5]. By repeating the shuffling experiments, highly effective genes can be obtained that display almost no homology to the original DNA templates. An alternative approach to creating functionally active synthetic genes is based on the screening of peptide libraries. This approach was used successfully to develop antimicrobial genes such as *D4E1*, which confers broad-spectrum tolerance to *Colletotrichum destructivum* if expressed in tobacco [6].

Glossary

Apomixis: asexual reproduction through seed.
CpG islands: regions devoid of methylation that have a higher C/G content than the genome average.
Heterosis: the increased fitness in hybrids over those of parents.
Inbreeding depression: reduced fitness in offspring over those of closely related parents.
Linkage drag: the reduction of fitness caused by genes that are inadvertently introgressed with genes controlling desirable traits.
Native DNA: DNA derived from plants that are sexually compatible with the target species.
Promoter trapping: identification of promoters through insertional mutagenesis with either T-DNAs or transposable elements carrying promoterless reporter genes.
Quantitative trait loci: loci associated with traits that are measured quantitatively rather than qualitatively.
RNA fingerprinting: PCR-based method to visualize differently expressed genes.
Somatic cell hybridization: hybrids derived from somatic cell fusions.

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Additional genetic elements that are needed for stable transgene expression also generally represent foreign or synthetic DNA. For example, the T-DNA that is used for transfer and stable integration of foreign DNA into plant genomes is derived from the tumor-inducing *Ti* plasmid of *Agrobacterium*. Some of the most widely used foreign regulatory elements include the 35S promoter of cauliflower mosaic virus and the transcriptional terminator of the *Agrobacterium* nopaline synthase gene, which promote high-level gene expression in transgenic plants. Examples of synthetic elements include chemical-inducible and pathogen-inducible promoters that contain multiple copies of specific transcription-factor binding sites [7,8]. On average, transgenic plants approved for commercialization contain ten genetic elements that are either isolated from foreign sources or represent synthetic DNA (http://www.agbios.com/ contains a database of genetically modified plant products). A typical example of a construct inserted into a plant genome for commercial purposes is the NewLeaf Plus® potato variety (Monsanto; http://www.monsanto.com/monsanto/layout/default.asp), created for dual resistance to the Colorado potato beetle and potato leaf roll virus [9] (Figure 1a).

Public debate

Public dialog about genetic engineering was triggered with the launch of transgenic varieties in the mid-1990s. Although scientific assessment points to no unique risks from genetically engineered crops, the absence of consumer benefits resulted in a widespread rejection of transgenic foods [10]. Multiple studies have shown that the extent to which transgenic organisms differ from traditionally bred organisms underlies much of the controversy surrounding the use of genetically engineered organisms [11–13]. A recent market survey in Mississippi showed that 81% of respondents would eat a vegetable with an extra gene from the same vegetable whereas only 14% would eat that vegetable if it had an extra gene from a virus [14]. In the face of this public perception, only the acreage of transgenic crops destined for feed, oil, fibers and processed ingredients has increased over the past decade; transgenic products closer to the table, such as fruits and vegetables, have been hindered in their development [15]. Rejection of the NewLeaf Plus® variety by processors and consumers prompted market withdrawal within a year of launch, and fear of consumer rejections also resulted in the recent shelving of herbicide-tolerant spring-planted wheat [16].

To allow more conscious differentiation in the perception of genetically engineered foods, a categorization of the associated crops was recently proposed [13]. According to this proposal, the introduction of foreign DNA creates 'transgenic' plants, whereas 'xenogenic' plants result from the insertion of laboratory designed DNA for which no naturally evolved genetic counterpart can be found or expected. Some of these two groups of plants deviate substantially (genetically, biochemically and physiologically) from what has been achieved through conventional breeding [13]. By contrast, rearrangements of genomic material from within the same sexual compatibility group would create 'intragenic' plants. Such modifications would often alter traits in a similar but more efficient and precise manner than that of plant breeding.

Use of native genes for crop improvement

Over the past decade, rapid advances in plant molecular biology resulted in a major shift from bacteria and viruses

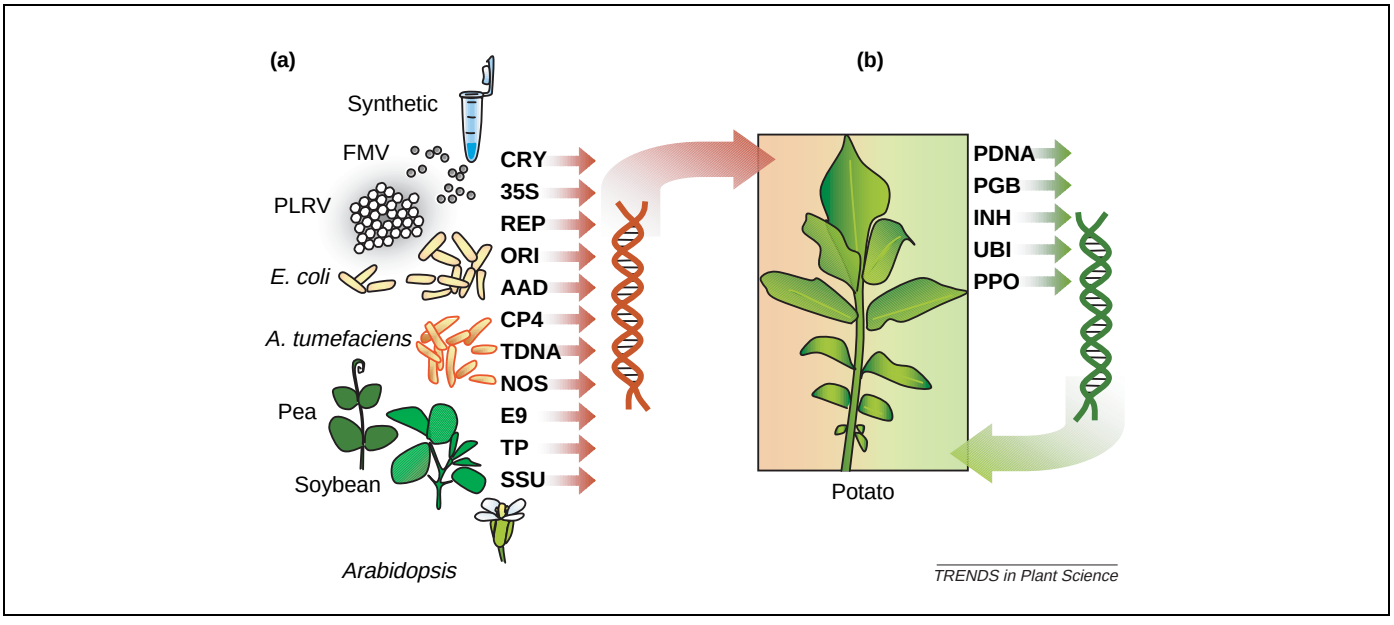

Figure 1. DNA sources used to create two genetically engineered potato varieties. **(a)** NewLeaf Plus® contains one synthetic gene and ten foreign genetic elements derived from figwort mosaic virus (FMV), potato leaf roll virus (PLRV), *E. coli*, *Agrobacterium tumefaciens*, pea, soybean and *Arabidopsis*. **(b)** Intragenic Russet Ranger plant containing five genetic elements from either potato or a sexually compatible wild relative. Abbreviations: 35S, 35S promoter of figwort mosaic virus; AAD, spectinomycin resistance gene from *E. coli*; CP4, *Agrobacterium EPSPS* gene; CRY, synthetic gene encoding a protein identical to the *Bacillus thuringiensis* Cry3Aa protein; E9, terminator of the pea Rubisco small-subunit gene; INH, potato vacuolar invertase inhibitor gene; NOS, *Agrobacterium* terminator of the nopaline synthase gene; ORI, origin of replication of bacterial plasmid pBR322; PDNA, transfer DNA from wild potato; PGB, promoter of potato granule-bound starch synthase gene; PPO, potato polyphenol oxidase gene; REP, PLRV replicase gene; SSU, promoter of the *Arabidopsis* Rubisco small subunit gene; TDNA, *Agrobacterium* transfer DNA; TP, transit peptide sequence of the soybean *CTP2* gene; UBI, terminator of potato ubiquitin-3 gene.

to plants as important gene sources. A broad variety of plant genes associated with agronomically important traits have now been identified, and continued developments in genomics applications are likely to further accelerate this gene discovery process [17]. For instance, herbicide tolerance has been associated with point mutations in native target genes. Plants containing modified acetolactate synthase (ALS) genes displayed the same high levels of sulfonylurea tolerance as transgenic plants that expressed bacterial ALS tolerance genes [18]. Likewise, the occurrence of glyphosate tolerance in a goosegrass (*Eleusine indica*) biotype has been associated with a mutated 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene [19]. Importantly, an understanding of gene modifications that are required for enhanced herbicide tolerance in specific plants can be readily applied to any target crop.

For disease control, the application of transposon tagging and map-based cloning methods resulted in the isolation of more than 50 functionally active resistance (R) genes [20], several of which are currently being used as valuable alternatives to foreign antimicrobial genes in crop improvement programs. One of the most agronomically important isolated R-genes is the *Solanum bulbocastanum RB* gene, which provides resistance to the potato late blight fungus *Phytophthora infestans*. The availability of this gene makes it possible to avoid INBREEDING DEPRESSION and LINKAGE DRAG for the production of resistant potato cultivars that are acceptable to the industry [21]. Transgenic approaches can also be used to broaden the spectrum and durability of resistance by stacking the *RB* gene with other isolated potato disease resistance genes.

Although synthetic *Bt* genes still provide the most effective means of controlling specific lepidopteran and coleopteran insects, rapid progress has been made in the development of plant-based gene alternatives. Protein analyses of plant extracts uncovered various insecticidal proteins, some of which provide protection against insects if overexpressed in transgenic plants. For example, a 30-kDa maize cysteine protease can be used to enhance tolerance to caterpillars and armyworms in maize [22]. Another approach enhances the plant's ability to produce known insecticidal secondary metabolites by either silencing or overexpressing key biosynthetic genes. In tobacco, suppression of a P450 hydroxylase gene resulted in a 19-fold increase of cembratrieneol levels in trichomes, dramatically enhancing aphid resistance [23]. Furthermore, hornworm tolerance was obtained by increasing tryptamine levels through overexpression of tryptophan decarboxylase [24]. Additional resistance genes might be isolated through detailed molecular genetic analyses of insect resistance loci associated with glucosinolate profiles [25].

Perishable foods undergo multiple changes during storage, some of which can negatively affect their quality. Such changes can often be countered by expressing sense and/or antisense fragments of genes associated with biochemical pathways controlling these changes in plants. For example, silencing methods were successfully used to increase shelf life in tomato. Downregulation of the polygalacturonase (PG) gene resulted in a reduced breakdown of pectin, thus slowing cell wall degradation, delaying softening and enhancing viscosity characteristics [26]. Although technical, financial and legal issues resulted in withdrawal of the original transgenic tomato variety, application of the PG technology in fresh market and processing tomatoes is still justifiable from a commercial point of view.

Another effective approach to improving storage characteristics of food crops relates to antisense expression of the starch-associated *R1* gene in potato. The resulting reduced accumulation of glucose and fructose limits non-enzymatic browning during cold storage [27]. Similar methods for the silencing of polyphenol oxidase genes made it possible to eliminate enzymatic browning, a process that results in the bruising-induced blackening in crops such as apple, lettuce and potato [28].

About 40 diverse plant genes have been used to enhance the ability to tolerate abiotic stresses [29]. Some of the most effective and thoroughly studied strategies are based on overexpression of the C-repeat-binding transcription factor (CBF) genes. In *Arabidopsis*, CBF overexpression triggers significantly increased survival rates to freezing, drought and salt [30]. The presence of stress-induced homologs in other plants such as rice [31] implies a broad applicability of CBF technologies.

Protecting the plants against damaging effects of free radicals can also enhance stress tolerance. Overexpression of plant ferritin genes prevents damage from free radicals produced by iron–dioxygen interactions and also results in a beneficial increase of iron content in transgenic crops [32]. Another way of improving plant productivity and human health is by increasing levels of carotenoid pigments such as zeaxanthin and lutein, which dissipate thermal energy and function as antioxidants that have been identified as possible protective agents in human vision and immune function, and in the prevention of cancer and heart disease [33]. In tomato, the metabolic flux to zeaxanthin was increased by simultaneously overexpressing the lycopene-β-cyclase and β-carotene-hydroxylase genes [34]. Antioxidant levels can also be increased by overexpressing native genes that encode rate-limiting enzymes or regulatory proteins for the biosynthesis of carotenoids, flavonoids and anthocyanins such as phytoene synthase [35], chalcone isomerase [36] and the transcriptional regulator ANT1 [37]. Furthermore, levels of the antioxidant vitamin C were increased by either overexpressing the wheat dehydroascorbate-reductase gene in tobacco [38] or the strawberry D-galacturonic acid reductase in *Arabidopsis* [39]; overexpression of the barley 4-hydroxyphenylpyruvate dioxygenase gene increased vitamin E levels in tobacco [40]. Experiments are under way to confirm that similar increases can be obtained by overexpressing these genes in their native backgrounds.

Efforts to reduce the amounts of antinutritional compounds through silencing approaches have been equally effective. Glycoalkaloid levels were reduced twofold in potato by antisense expression of a solanidine UDP–glucose glucosyltransferase gene [41], and cassava cyanogens levels were lowered by simultaneously targeting two

cytochrome P450 genes [42]. Furthermore, sense suppression of the soybean papain protease gene resulted in elimination of one of the three main seed allergens [43], and the resulting transgenic line can be crossed with available mutant lines lacking other major soy allergens to produce hypoallergenic soybean.

One of the greatest opportunities for genetic modification of plants is enhancement of taste. The genetic complexity of traits associated with taste and difficulties in quantifying it have hampered efforts to isolate key genes. However, recent isolation and characterization of key genes in flavor biogenesis, such as the citrus valencene synthase gene [44], and the strawberry alcohol acyltransferase [45] and *O*-methyltransferase genes [46], provide the first tools for metabolic engineering. Continued developments in plant molecular biology will increase our ability to develop genetically modified plants that contain new traits. It might be possible not only to enhance plants' stress tolerance and improve their health and taste characteristics but also to engineer important and still poorly understood processes such as HETEROSIS [47] and APOMIXIS [48].

Native regulatory elements

Various methods (including PROMOTER TRAPPING and RNA FINGERPRINTING) have facilitated the isolation of hundreds of plant promoters. Many of these regulatory elements, such as the promoters of ubiquitin and actin genes, support high-level gene expression in most tissues of transgenic plants [49,50], whereas others can be used for the precise, tissue-specific or inducible expression of new traits. Even more options might be provided by methods that use a computational approach for promoter detection. This approach is based on the identification of *cis*-acting regulatory elements and specific characteristics of promoter-associated CPG ISLANDS [51].

Functional polyadenylation signals are available for most major crop species to terminate transcription. Some of these elements, such as the 3′ sequences of the ribulose-1,5-bisphosphate-carboxylase (Rubisco) small-subunit gene were shown to direct high expression levels in plants [52]. Other plant genetic elements that can be used to regulate gene expression and/or stabilize transcripts include plant introns, untranslated leaders and the ubiquitin-monomer-encoding sequence.

Shunning foreign markers

After the initial transformation and regeneration process is complete, selectable marker genes are not required for transgene expression. Therefore, methods were developed that allow removal of such markers from plant genomes. One strategy that has been applied successfully in the tobacco model system includes positioning the marker gene between recombination sites so that it can be excised from the plant genome using an inducible recombination system [53] (Figure 2a). However, the complexity of this method limits its applicability and resulting marker-free plants still contain one of the recombination sites derived from bacteriophage P1. An alternative and more effective method places the marker within a second T-DNA, whereby it can be physically segregated and lost in subsequent progeny [54] (Figure 2b). Unfortunately, marker-removal methods are labor intensive and often too inefficient to allow their widespread use in commercial product development programs [3], particularly in asexually reproducing or vegetatively propagated crops and for cases in which many primary transformation events are required.

It is also possible to use marker-free transformation systems. Early methods excluded a selection step and are inefficient, with transformation frequencies of 0.2% in potato. However, these frequencies can be increased to up to 5% by applying supervirulent *Agrobacterium* strains [55] (Figure 2c). Even more efficient methods were developed by using *Agrobacterium* strains harboring two different transfer DNAs, one carrying a positively and a negatively selectable marker gene and the other containing the DNA of interest. A transformation procedure including a transient positive selection step for marker gene expression followed by a negative selection step for marker gene integration yielded plants containing only the desired DNA with frequencies of ~29% [28] (Figure 2d).

A final method can be used only in plants that are accessible to plastid transformation, such as tobacco, tomato and potato. This method transforms mutants with constructs that contain reconstitution elements and a gene of interest cloned within the flanks used for homologous recombination, and a marker gene outside of these flanks [56]. Upon removal of selection pressure, 'loop out' recombination results in the loss of co-integrates, thus giving rise to marker-free plastome transformants containing only the gene of interest (Figure 2e).

Recent studies show that some plant genes can themselves be used as transformation markers. The most interesting native marker to date is a modified protoporphyrinogen oxidase gene. Maize transformants expressing this gene were produced via butafenacil selection using a flexible light regime to increase selection pressure [57]. Successful tobacco chloroplast transformation with a spinach betaine aldehyde dehygenase gene [58] suggests that native genes involved in the conversion of betaine aldehyde can also be used as markers for plant transformation. Several additional native markers function effectively but trigger cytokinin responses, which confer an undesirable phenotype on the transformed plant [59]. Upon transformation, such plant markers must still be removed by any of the methods described above.

Reduced backbone integration

The DNA transferred from *Agrobacterium* to plant cells was originally assumed to comprise only the T-DNA region. However, multiple studies have shown that transferred DNA often includes additional sequences of the binary vector. This superfluous 'backbone' DNA contains bacterial genetic elements including origins of replication and antibiotic resistance genes. The presence of such DNA in the genomes of transgenic plants is, therefore, undesirable. Backbone integration frequencies range from 50% to 85% in solanaceous species such as tobacco, tomato and potato [60,61] and from 38% to 62% in plants such as *Arabidopsis* [62] and rice [63]. Efforts to

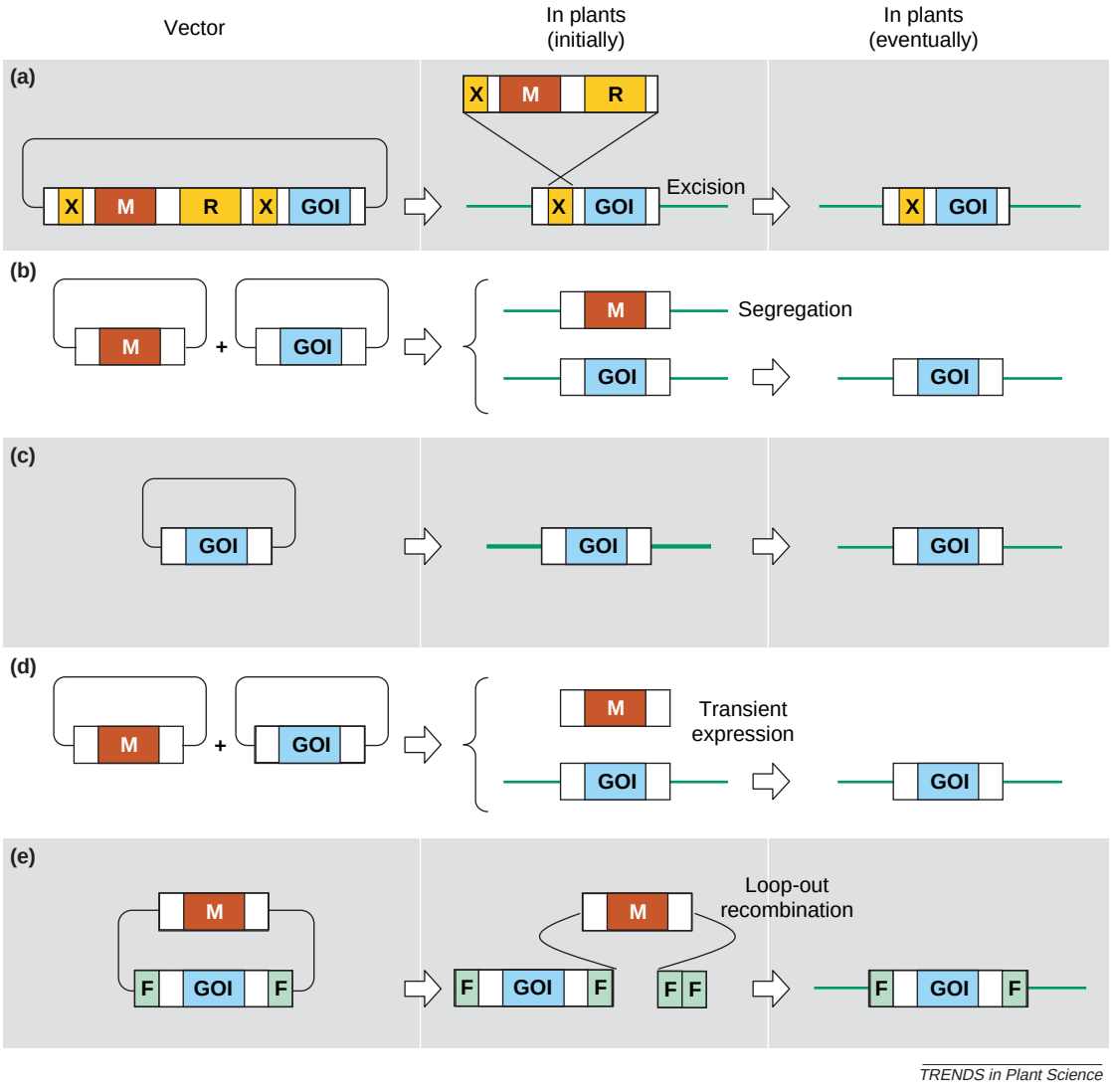

Figure 2. Methods to generate marker-free transformed plants. **(a)** An expression cassette for a selectable marker gene (M) is placed, together with an expression cassette for a recombinase (R), between recombination sites (X). Upon transformation, expression of the recombinase gene might result in excision of the region between the recombination sites, thus enabling the recovery of transgenic plants only containing an expression cassette for the gene of interest (GOI) and a single remaining recombination site. **(b)** Co-transformation of plants with both a T-DNA vector carrying the selectable marker gene and a vector containing the GOI might result in unlinked integration events, which can be segregated in progeny plants. **(c)** T-DNAs lacking M can be used to generate transformed plants infrequently. **(d)** Co-transfer of two T-DNAs, one carrying M and the other containing the GOI, followed by a transient selection step results in the frequent integration of only the GOI T-DNA. **(e)** Plastid transformation using a vector in which M is cloned outside the homologous flanks. The marker co-integrates via a first recombination but is excised upon a subsequent loop-out recombination event.

reduce the frequency of transgenic plants containing backbone sequences have been based mainly on the use of *Agrobacterium* plasmids that contain a marker gene outside the T-region. Use of the barnase gene made it possible to select against backbone integration events [61], whereas cytokinin markers such as the *Agrobacterium* isopentenyl phosphotransferase gene were used successfully to screen against such events [28].

All-native DNA transformation

Until recently, molecular strategies and gene choices might have limited, but did not eliminate, foreign DNA introduced into a plant. An example of a genetically modified plant containing only a few foreign genetic elements was produced by plastid transformation. This tobacco plant carries the β-glucuronidase gene from *E. coli* fused to a native chloroplast promoter and followed by a terminator derived from *Chlamydomonas* [56]. Another plant with only a small amount of foreign DNA is an amylose-free potato plant produced through marker-free transformation [55]. This plant contains the granule-bound starch synthase gene driven by its native promoter and followed by the terminator of the nopaline synthase gene inserted within a T-DNA. Only the terminator and T-DNA represent foreign DNA. Although a potato terminator could have easily replaced the bacterial terminator, plant-derived alternatives for the *Agrobacterium* T-DNA were not available until recently.

Plant transfer DNAs (P-DNAs) were uncovered through database searches and PCR analyses for sequences that resemble T-DNA borders. In spite of some sequence divergence, P-DNAs from various plant

species, including potato and tomato, have been shown to support DNA transfer from *Agrobacterium* to plant cells [61] (C.M. Rommens, unpublished). A potato P-DNA supported almost twice the potato transformation frequency that a conventional T-DNA did [28]. This P-DNA was used to introduce a vacuolar invertase inhibitor and a modified polyphenol oxidase gene into potato (Figure 1b). The resulting plants were shown to display reduced cold-induced sweetening and black spot bruise tolerance. In accordance with the recently proposed classification of genetically modified organisms, these all-native DNA plants can be termed 'intragenic' [13].

Possible issues associated with all-native DNA transformation

The use of plant genes for the incorporation of new traits might not always be as effective as procedures that rely on foreign or synthetic genes. As discussed above, bacterial sources still provide the strongest genes for glyphosate and insect tolerance. Other examples involve viral pathogens, some of which can be controlled most effectively with viral genes. Although plant-based strategies for all targets will continue to improve, the value of extremely strong 'super' traits is still untested. High herbicide tolerance levels can encourage excessive herbicide usage and hasten the selection of tolerant weeds. Furthermore, such super traits might increase the negative ecological consequences of inadvertent gene flow [64].

Another issue is that native genes are in some cases more difficult to overexpress than foreign genes. For instance, genes regulated by negative feedback mechanisms, such as the threonine synthase and aspartate kinase genes, must be modified to become feedback insensitive [65]. It is also possible for the introduction of a new gene copy to trigger sense suppression responses [66], particularly in polyploid plant species; this can be countered by linking gene fusions to matrix-associated regions that shield the introduced gene from RNA silencing [67].

A third issue is that engineered silencing of certain undesirable genes can be inactivated by silencing suppressors produced by infecting viral pathogens [68]. Molecular strategies to prevent virus-induced silencing or suppression need to be directed towards a further understanding of how viral suppressors interact with plant proteins. However, infection-induced silencing suppression might be beneficial because it would emphasize the need for seed certification.

Finally, all native plant genes considered for overexpression should be evaluated carefully for their safety. Genes that encode proteins stable to digestion, such as most antimicrobial cysteine-rich peptides, and proteins that resemble known allergens should be excluded because their expression might induce new food sensitivities.

Engineering and breeding

By using the same genetic material available to plant breeders, genetic engineering approaches can be more readily integrated into existing plant breeding programs. Instead of overriding the plant breeding approach by incorporating foreign DNA and developing super traits, genetic engineering would become just one of the many tools that are available to modern breeding. Indeed, most traits developed through all-native DNA transformation methods might also be developed through plant breeding. However, the process would generally take more time to accomplish, particularly if multiple traits were targeted simultaneously. Furthermore, most traits introduced through genetic engineering are dominant and can be moved from one plant to the other as a single locus. Another advantage of transformation is that it results in the modification of a few well-defined genetic elements, whereas sexual recombination might lead to the introgression of undesirable genes involved in the production of allergens or toxins, if these genes are linked to the traits of interest [69]. The risk of unwittingly introducing such genes into food supplies is increased through efforts to untap the genetic diversity of distant relatives that have not been used before for food consumption [70]. Given the projected doubling in global food demand over the next 50 years and the prediction that yield gains will be more difficult to achieve [1,71], only fully integrated crop improvement programs will provide the sustainable agriculture that is needed. These efforts can be expected to result in the first products derived from intragenic crops within the next five years.

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