14
Design of Safe and Biologically Contained Transgenic Plants: Tools and Technologies for Controlled Transgene Flow and Expression

YURI GLEBA*, SYLVESTRE MARILLONNET AND VICTOR KLIMYUK

Icon Genetics AG, Biozentrum Halle, Weinbergweg 22, D-06120 Halle/Saale, Germany

Introduction

Plant biotechnology as a commercial process is a reality. Data released by USDA and ISAAA show that in 2002, the global GM crop area continued to grow for the sixth consecutive year, and reached 58.7 million hectares, or 145 million acres. In 2003, 81% of soybeans, 73% of cotton and 40% of maize grown in the USA were genetically modified. Such numbers undoubtedly reflect benefits enjoyed by the various participants in the business, including some 5.5–6 million farmers. All GM plants grown at present were modified by incorporation of input traits that facilitate crop production. GM plants expressing output traits are also being developed. One example is the development of transgenic host plants for the production of pharmaceutical proteins. Although there are currently no plant-made biopharmaceuticals on the market, several companies have announced Phase II–III clinical trials of 'biogenerics', and some antibodies and enzymes; thus, it is simply a matter of time before such products are approved and appear in our pharmacies.

Fears of, and resistance against, plant biotechnology-based business are a reality, too. Reviews, research papers, editorials, and published 'letters to the editor' dealing with safety and regulatory issues relating to transgenic plants make up a significant portion of publications in news media and major scientific journals. The dialogue is active, often emotionally charged, and involves diverse members of society. Governmental and epi-governmental regulators all over the world are busy defining the

---

*To whom correspondence may be addressed (gleba@icongenetics.de)

Abbreviations: GM, genetically modified; GMO, genetically modified organism; USDA, United States Department of Agriculture; ISAAA, International Service for the Acquisition of Agri-biotech Applications.

Biotechnology and Genetic Engineering Reviews – Vol. 21, November 2004
0264–8725/04/21/325–367 $20.00 + $0.00 © Intercept Ltd, 9 Farriers Close, Colehill, Wimborne, Dorset, BH21 2UA, UK

legislative framework for safety rules and restrictions that would satisfy both their electorate and businesses, while remaining scientifically wise.

Covering all aspects of this ongoing dialogue is beyond our capabilities. The reader is referred to numerous recent publications that review different aspects of GM plant biosafety, such as the potential environmental impact of transgenic crops (Dale *et al.*, 2002), analysis of the potential impact of transgenic plants on human health (Conner and Jacobs, 1999; Falk *et al.*, 2002; Lack, 2002), regulatory approaches covering the release of transgenics in the US and EU (Nap *et al.*, 2003), ethical aspects of plant genetic engineering (Ellstrand, 2003), proposed initiatives of professionals in the area of GM plant biosafety (Kapuscinski *et al.*, 2003), and, last but not least, analysis of the technologies that might be used for gene containment (Daniell, 2002; Wickelgren, 2003).

The topic of this review is *'safety and control that can be derived from safe technology rather than from legal regulations'*. Our approach, which we tried to apply consistently in this analysis, is that of an 'engineer', i.e. a technologist, whose task is to develop a process that satisfies the requirements of an exploiter (technical efficiency, compliance with business requirements, compatibility with existing or predicted infrastructure), a governmental regulator (regulatory compliance, sustainability), and an end user. Moreover, we are also trying to act as an 'ethical engineer', who is using the latest science and technology tools to design not just a more efficient process, but also a safer, more ecologically benign and sustainable one. Such process has to exceed currently legislated safety thresholds and, ideally, be able to satisfy future safety concerns. Being unable to predict future legal requirements, we chose to rely entirely on safety that is generated by underlying technology, and that can be designed through the use of safe technical components, and by building safe protocols using those components.

General considerations

NO 'ONE SIZE FITS ALL'

Analysis of the safety issues in plant biotechnology is difficult because it has to reflect multiple elements, some of which are not entirely known/developed at present, and multiple scenarios and processes, most of which are not even in existence today. Plant biotechnologies represent a potentially powerful new technology platform for multiple businesses, including agriculture, food, feed, specialty chemicals, human and animal health, etc., the broad applicability and the nascent nature of which are again complicating the analysis. One can compare the problem with the safety issues in an existing, mature industry: transportation. Although based on the same or similar technology principles, the tools and processes in the automobile and aircraft industries have been safety-optimized in very different ways. It is obvious that the safety issues relating to transgenic plants engineered to be insect-resistant will be different from those relating to plants engineered to express pharmaceutical proteins. It therefore makes sense to broadly define classes of genetically modified plants that could be produced under broadly similar production processes, and would require similar 'safety locks and circuits'. For the purpose of this review, the following four classes are proposed (*Table 14.1*):

Table 14.1. Broad categories of GM plants requiring different biosafety levels

I.	Plants with improved 'input traits', such as pest control (disease resistance, nematode resistance, herbicide tolerance), yield enhancement, including abiotic stress tolerance, improved plant nutrition and water use.
II.	Plants intended for food or feed with improved 'output traits' present in 'physiologically safe' amounts, such as improved nutrients (protein, amino acid, oil, carbohydrates, polysaccharides composition and quality; phytochemicals, e.g. antioxidants, isoflavones; micronutrients, e.g. vitamins, iron, etc.), reduced allergenicity, reduced waste, extended shelf-life, improved processing and consistency, etc.
III.	Plants for food/feed with output traits that are present in high ('non-physiological') amounts, which have to be processed and adjusted for the amount of active ingredient before use as a food/feed.
IV.	Plants *not intended for food or feed*, such as production hosts for pharmaceutical proteins or industrial enzymes.

Examples of the first class are commercialized corn, cotton, soybean, and canola varieties that are resistant to herbicides or insects. Examples of the second class are plants with elevated vitamin content ('golden rice'), or tomato varieties with extended shelf-life. Among the class III plants, we would consider canola varieties that currently are being engineered for high expression levels of omega-3 fatty acids, high levels of phytase, or plants expressing high amounts of sweet proteins such as thaumatin. It is obvious that plants of classes III and IV require a much higher level of biological separation and control than the plants that are safe as a food, and much of this review discusses technical solutions that are appropriate for such plants. Unlike plants of classes I and II, plants of classes III and IV are intended for intensive production of highly active ingredients, and have to compete on the market with other production hosts, such as bacteria, yeasts, fungi, transgenic animals, and cell cultures. For such production hosts, we will need constructs more complex than the regular expression constructs that typically contain a gene fused to a strong constitutive promoter.

SAFETY IS 'ONLY' AN IMPORTANT COMPONENT OF A 'PACKAGE DEAL'

Although we often declare that 'safety cannot be compromised', in a real world, we settle for a calculated risk and accept certain 'benefit v. risk bargains'. One of the most telling phenomena of our political reality is the fact that, despite the more than 1 000 000 annual deaths in automobile-related accidents worldwide, including over 100 000 in the US and EU alone (http://www.safecarguide.com/exp/statistics/statistics.htm), and the devastating effect of the transportation industry on the environment, there is no serious political opposition to this industry. In this case, the general public regards the price as being worth the benefits enjoyed. In the case of plant biotechnology, the same public can be much more negative, as the benefits at the level of an individual (other than the 6 million farmers who grow GM crops) are still hypothetical. This negative attitude is being effectively exploited by non-governmental organizations that thrive by selling technophobic fears. A businessman, on the other side, regards a plant biotechnology-based business as a worthwhile enterprise only if the cost of safety-related modifications of the production and distribution processes still allows for measurable profit margins. As a result, the

genetic engineer and the inventor have to find solutions that take into account these seemingly conflicting requirements, because only processes and scenarios that will accommodate all parties will have a chance to survive and become a commercial technology platform.

CERTAIN TECHNOLOGIES CAN BE SIMULTANEOUSLY MORE PROFITABLE AND SAFER

We mentioned profitability and safety requirements as being in seeming contradiction. A priori, a safer process often requires more complicated (and more expensive) technology solutions. Current advocates of 'keeping it simple' (to obtain regulatory approval more easily and to limit associated costs) should be reminded that aeroplanes at the beginning of the aviation era were much simpler than aircraft used today, but that today's machines are infinitely safer. It is certain (and it is assumed in this analysis) that future plant biotechnology processes will involve multiple genes and require complex regulation. A 'technically regulated process', i.e. a process in which an operator can deliberately and effectively interfere, becomes at some point inevitable for any industry, as it contains in itself multiple solutions: a safer process, a more efficient process, and a product and a process that are better controlled by the owner.

PRODUCT (SELF-)REPLICATION: IMPLICATIONS FOR BUSINESS AND SAFETY

Plants are 'self-replicating products'. Therefore, the plant biotech industry is faced with problems strikingly similar to those experienced today by other industries whose manufactured products can be easily copied, such as digital music and movies disks. Plant breeding companies have always tried to develop technologies that would limit the farmer's ability to replant part of harvested seed. The commercial success of corn as a crop is, to a great extent, the result of development of hybrid seed varieties, which limit the yield benefits of the purchased seed to a single generation, thus providing better control (and better profit margins) for the germplasm developer and the seed producer. With the advent of plant genetic engineering, agrochemical companies such as Monsanto have developed a business model that is not built around patented, and difficult to manufacture, chemical compound (pesticide) but, instead, relies on sales of a germplasm, a 'self-replicating product'. To successfully exploit this new technology, and to recoup the high costs invested in research and development, these companies now have to figure out how to prevent unauthorized/illicit replication and exploitation of their germplasm. Furthermore, the great pace allowed by this new technology, as well as the development of radically different new plants and products, threatens to contaminate existing traditional production processes and products, potentially leading to health risks, ecological hazards, and genetic pollution. The genetic engineer is therefore faced with a situation where powerful new products generated by high technology are produced using an 'old' industry infrastructure (open fields) that has, so far, only slightly been retrofitted to accommodate the 'new plants'.

YIELD AND SAFETY BENEFITS OF A BIOLOGICALLY REGULATED PROCESS

Another remarkable peculiarity of today's plant biotechnology industry is the absence of potential 'blockbuster' products in the R&D pipeline in the 'output traits' category. Except perhaps for omega-3 fatty acids, all other traits either have too small potential markets to appeal to large companies, or their development has been met with serious technical challenges. Examples of the latter type include phytase, modified oils, collagen, silk protein, polyhydroxybutyrates (biodegradable plastics), human serum albumin, etc. In most of these cases, expression of the trait of interest is not high enough to permit a competitive production process. Moreover, in those cases where a high level of expression can be achieved, agronomic performance/yield of the crop is also severely affected. The conflict between high level of expression of a gene of interest on one side, and growth and development of the plant expressing this gene on the other side, is a well-known phenomenon. This problem can, however, be overcome by separating the 'growth phase' from the 'production phase', as is often done in the microbial industry. In plants, some native or experimental proteins and molecules are being produced at very high levels, with up to 35% of total protein in a leaf (large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase), and up to 35% of total protein in a seed (single-chain antibody – Jaeger *et al.*, 2002), and there is no reason to believe that the same should not be possible with commercially useful products. Such high productivity will, however, most likely require more sophisticated expression controls than just using a strong constitutive promoter, as is the case with GM crops today. Thus, a more controlled process will not only bring safety benefits for consumers and the environment, but it will also provide a more efficient and productive protocol.

General review of relevant technologies

Uncontrolled spread of transgenes to non-transgenic plants and to wild relatives may have harmful consequences, both for humans and the environment. Consequently, the engineer's design of the technical process has to provide for a reasonable and controlled containment of genetically modified organisms, and for transgene flow control that will prevent escape of the transgene to non-modified plants of the same or related species. There are multiple barriers that one may consider, including mechanical, physical, ecological, geographic, temporal, regulatory, and, last but not least, biological. In real world situations, multiple barriers are likely to be used in a multi-layer safety system. In this analysis, we limit ourselves to reviewing the biological barriers only.

Several biological processes could be targeted when designing safety barriers, including the following:

(1) spread due to vegetative propagation;
(2) spread due to sexual reproduction;
(3) spread due to interspecific gene transfer, including transduction and transformation;
(4) expression of a transgene after (undesired) spread.

Below is a list of possible mechanisms that could be employed to create biological safety barriers:

Table 14.2. Possible components of biosafety control systems

- Sexual transmission control
  - Designated crop species
  - Vegetative propagation, e.g. apomixis
  - Cytoplasm control, male sterility, hybridization systems
- Transgene expression encryption
  - Multicomponent systems
  - Encrypted systems
- Control of transfer by transformation, transduction
- Epidemiological mistake correction
  - Controlled fitness of GM organism
  - Negative selection linked to the transgene
- Transgene tracking

Table 14.3. Genetic processes that could be a target of intervention or regulation with the aim of designing biosafety barriers and possible intervention 'switches'

- Transcription
  - Transcription repressor/activator, recombinase, integrase, flippase, resolvase, transposase
- RNA processing
  - Ribozyme, maturase
- Translation
  - tRNA, ribozyme, acetyl synthetase
- Post-translational
  - Intein
- Artificial enzymes, ribozymes, tRNAs, etc.

(1) For control of spread by sexual transmission: use of 'designated' crop species, use of facultative vegetative propagation, e.g. apomixis, use of male sterile lines, use of different 'locked' hybridizations systems.

(2) For control of gene expression, and for control of transfer by transformation or transduction: use of transgene encryption and of multi-component genetic systems.

(3) One would also need some tools to fight possible escape (correction of 'epidemiological mistakes'). For such a scenario, it would be useful to have systems that would allow control of the fitness of the GM organisms, or to apply negative selection on the transgene carriers.

(4) Finally, a mature system would also need an efficient system for tracking the transgenes, or transgene-containing and/or transgene-expressing organisms. This safety element has been given a lot of attention, with a variety of methods, based mostly on analysis of specific DNA sequences in tested material.

The summaries of possible control mechanisms and genetic processes that could be a target for designing biosafety barriers are presented in *Tables 14.2* and *14.3*, respectively.

CONTROLLED HYBRIDIZATION SYSTEMS

One of the potentially most useful technologies that may help to prevent the spread of transgenes to other varieties or to related wild species is *controlled sexual*

hybridization. Since a plant is a 'self-replicating product', and since reproduction of most crop plants is by sexual hybridization and seed formation, plant breeders have always been interested in techniques that would allow them to control sexual reproduction, either to stabilize and exploit an interesting individual genotype (by asexual reproduction), or, on the contrary, to limit expression of the trait of interest to one generation only (by hybrid seed systems).

Asexual reproduction: vegetative multiplication and apomixis

Stabilization of an interesting genotype can be achieved for perennial tree species by vegetative multiplication (for a review see Merkle and Dean, 2000). In such cases, sexual hybridization would be used only for breeding. Of course, mature plants obtained by this technology are fertile and capable of sexual hybridization, and therefore, this sort of control is not especially useful for our purpose.

One particular version of *de facto* vegetative multiplication is apomixis (Koltunow, 1993; Koltunow *et al.*, 1995; van Dijk and van Damme, 2000). Genetic control of apomixis is, however, far from being understood, and is probably polygenic. Some of the genes involved in the control of fertilization-independent seed development have been identified (Chaudhury *et al.*, 1997; Luo *et al.*, 1999). However, this did not bring us significantly closer to the possibility of engineering crops with apomictic reproduction mechanism. It is likely that many components will have to be integrated to produce apomictic seeds. In some species, apomictic propagation is accompanied by development of viable pollen that can fertilize non-apomictic plants (van Dijk, 2003), thus limiting usefulness of this system for transgene control.

Hybridization systems

Probably, most domesticated plants were originally capable of cross-pollination and had genetic mechanisms of self-incompatibility that favoured cross-pollination over selfing. During the numerous decades of practical breeding, which aimed at generating genetically uniform and high-performing populations, those self-incompatibility genes were bred out (for an example, in tomato, see Kondo *et al.*, 2002). Lines were also selected that were predominantly self-pollinating, thus giving rise to present lines that are often strict self-pollinators. Such inability to cross-pollinate is an excellent genetic barrier that should be used by a genetic engineer. It is obvious that transgenic varieties of soybeans, for example, are much more contained than transgenic canola lines, because soybeans, unlike canola varieties, are facultative self-pollinators (Rieger *et al.*, 2002). Such a 'free ride' is, however, not possible for many crops that retain a high level of cross-fertilization, such as corn, canola, sugar beets, barley, rice, etc. More importantly, for the purpose of genetic manipulation, an engineer can extract much more efficacy and safety from a system using controlled hybridization, since crossing plants is by far the easiest and best 'process switch' that we can have at our disposal. Much of our analysis here is based on the assumption that, for effective control, a two- or multi-component transgene system will be required, and that much of that control can be exerted by sexual crossing of two different plants.

There are many systems in use today for the production of hybrid seed. They

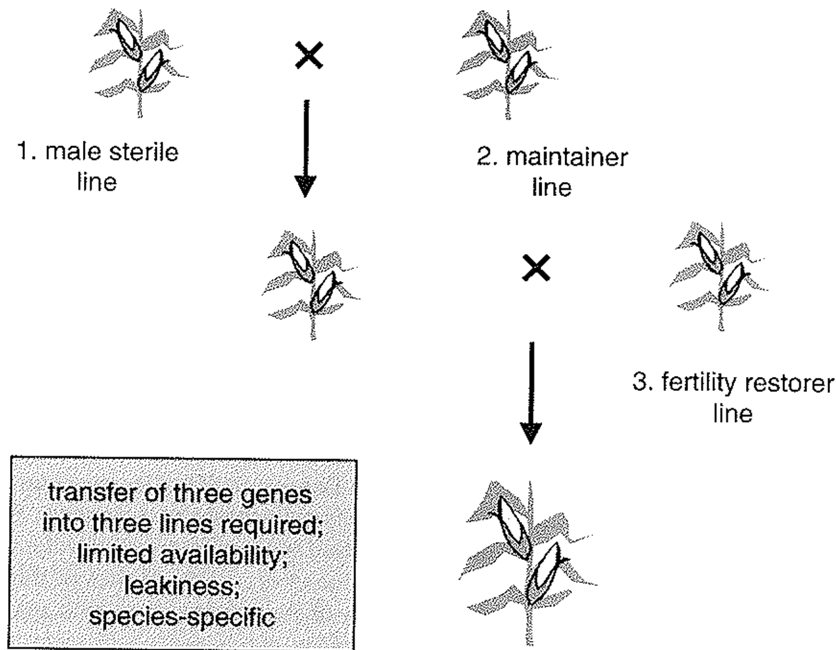


Figure 14.1. General scheme of hybrid seed production based on genetic male sterility. Note the need for at least three genetic components in three parental lines: male sterility genes in the male sterile line, sterility reversal genes in the male sterility maintainer line, and fertility restorer genes in the third parental line.

include systems based on mechanical castration, nuclear or cytoplasmic male sterility (developed by breeders), and transgenic male sterility. The classical genetic systems based on nuclear or cytoplasmic male sterility have been used for decades, but they are complex, and suffer from numerous problems. Male sterile phenotypes were discovered as a result of random selection or by interspecific hybridization. For their propagation, one needs a so-called 'maintainer line'. Also, in most crops, the sterility phenotype has to be fully reversed in the resultant hybrid, bringing up the need for 'fertility restorer genes'. All existing traditional methods in use today (for corn, rice, tomato, etc.) are based on two- or three-component genetic systems that are difficult to introgress and to maintain (*Figure 14.1*). Also, they do not always perform as expected, and cause additional expense for hybrid seed production.

Several companies have had projects aimed at developing transgenic systems for hybrid seed production. To our knowledge, only one has been commercialized so far. We refer here to canola hybrid seed technology that has been developed by Plant Genetic Systems, and that is used today for the commercial production of canola seed (Williams and Leemans, 1998). The system (*Figure 14.2*) is based on male sterility that is achieved by expression of the toxic enzyme barnase under control of a tight tapetum-specific promoter. For maintenance of sterile lines, expression of an additional gene, barstar, is required to reverse the toxic effect of barnase (Botterman *et al.*, 1996).

A different approach proposed by Burgess *et al.* (2002) and Werner *et al.* (2003)

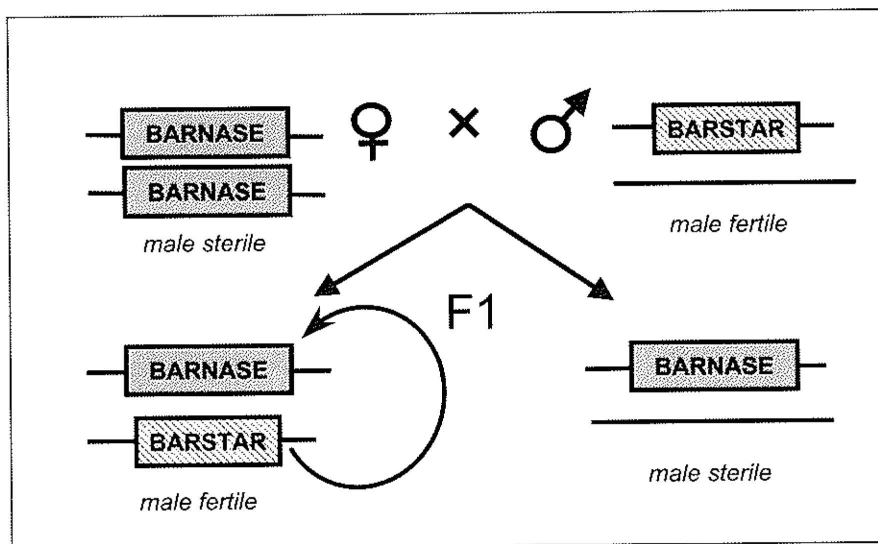


Figure 14.2. Male sterility and sterility maintainer genes used in transgenic hybrid seed system developed by Plant Genetic Systems (Williams and Leemans, 1998). The system relies on male sterility caused by a highly toxic ribonuclease, barnase, expressed under a tapetum-specific promoter, and reversal to fertility as a result of expression of barstar gene.

may represent a useful system for generation and maintenance of male sterile plants and for hybrid seed production. The process relies on co-expression of two pairs of genes coding for non-functional barnase fragments (or any other toxic protein) that cause male sterility in anther tissues when both protein fragments are present. Assembly of a functional protein is achieved by intein-based *trans*-splicing (see the paragraph 'Trans-*splicing inteins: reconstitution of functional protein from non-functional fragments*' below), but functionality can also be achieved by simple co-expression of separate polypeptides. In our work, for maintenance of the male sterile parent, the genes expressing the barnase fragments are linked to a second pair of genes expressing fragments of a herbicide resistance gene (acetolactate synthase). The two pairs of complementary genes are placed in isoallelic position (see also the paragraph '*Relative position of genetic components on a chromosome*' below) on homologous chromosomes (*Figure 14.3*), thus allowing for easy selection of male sterile 'heterozygotes' as plants that are resistant to the herbicide.

The hybrid seed systems developed by plant breeders were designed to ensure consistent and improved yield for one generation only. The seeds that are harvested by the farmer are fully capable of further sexual reproduction (with the exception of sterile triploid sugar beets that are obtained by hybridization between tetraploid and diploid parents). For our purpose, the hybrid seed systems described above are used for two other reasons: 1) one of the parents is male sterile, thus avoiding spread of the transgene via pollen (provided the transgene of interest is encoded in the male sterile parent only); and 2) more importantly, the system is used as an effective switch to turn product synthesis on, and, if necessary, to simultaneously render the resulting progeny biologically sterile, i.e. incapable of subsequent sexual reproduction.

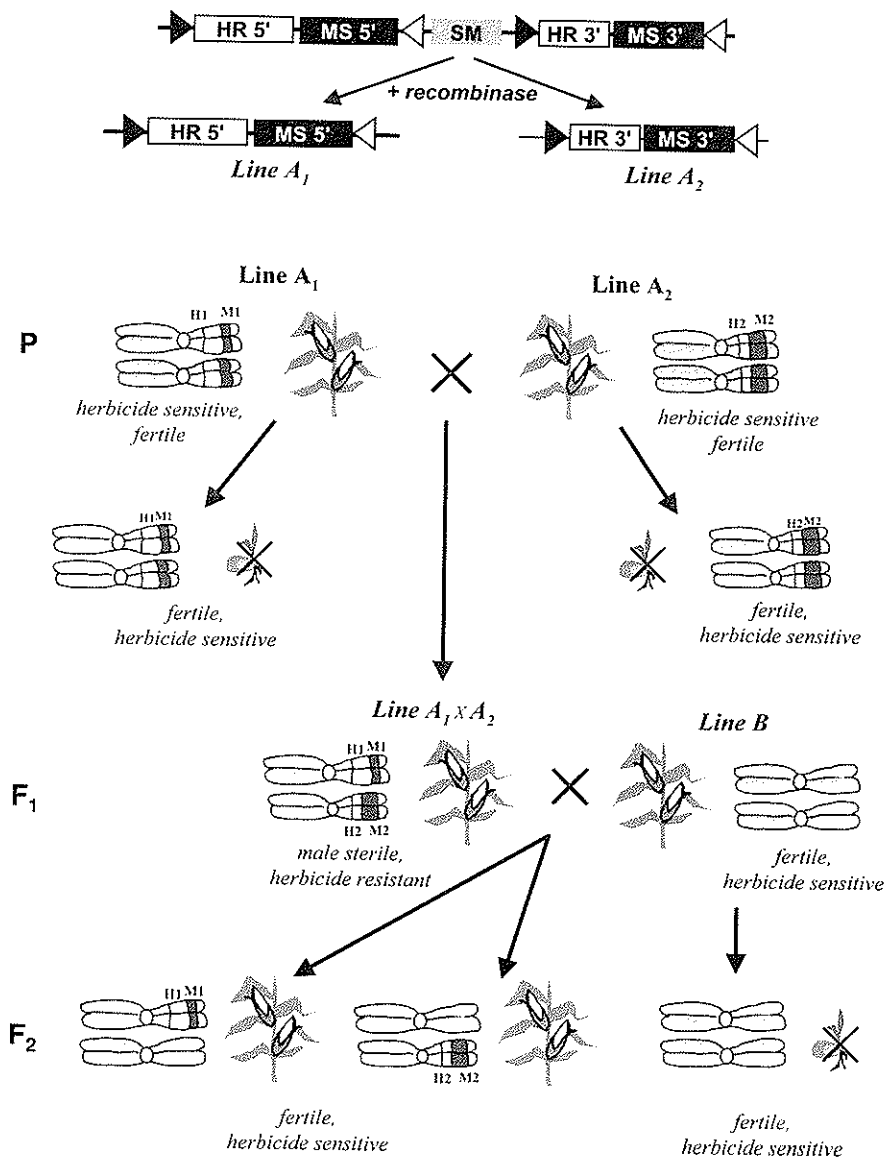


Figure 14.3. Hybrid seed technology utilizing pairs of genes encoding fragments of proteins that require intein-mediated *trans*-splicing (Werner *et al.*, 2003). The system relies on two pairs of genes encoding complementing protein fragments capable of *trans*-splicing and providing for two linked phenotypes expressed in a heterozygous plant only: male sterility and herbicide resistance. The heterozygous male sterile parent can be created and maintained by selecting for herbicide resistance; in the commercial hybrid seed progeny, fertility is fully restored.

Hybridization control relying on maternal inheritance

In the previous paragraph, we mentioned cytoplasmic male sterility as a component of hybrid seed systems. In a plant cell, in addition to the nucleus, which contains

99% of the genetic material, two other organelles (plastids and mitochondria) also have their own genetic systems. In many important crops, plastid genes are transmitted strictly maternally. Therefore, introducing transgenes into organellar DNA rather than in the nucleus offers a level of safety by preventing unwanted spread of transgene through pollen. Plastid-encoded transgenes also offer the advantage of high levels of gene expression (for a review see Maliga, 2003a). At present, however, there are several limitations to this approach:

(1) despite more than a decade of efforts by numerous groups, plastids of only one species, tobacco, can be transformed routinely;
(2) there are no reliable methods of genetic transformation of mitochondria;
(3) proteins expressed from organellar DNA are confined to the organelle and are not glycosylated; and
(4) like most other genetic systems, paternal exclusion is not absolute, i.e. transfer of organelle genes via pollen can be detected at low frequency; and, in addition, also with very low frequency, the transgene can 'escape' from the plastid to the nucleus (Daniell and Parkinson, 2003; Huang *et al.*, 2003; Maliga, 2003b; Stegemann *et al.*, 2003), and thus overcome its confinement.

ENCRYPTION SYSTEMS AND GENE SWITCHES

In an attempt to create a system that gives us reliable control over transgene transmission ('germplasm control') and/or expression ('expression control'), we inevitably come to a scenario that requires at least two or more components. Depending on the configuration of the components, the system acquires the possibility to be in two states: 'on' and 'off'. These configurations are designed and implemented through the use of molecular genetics tools, and proper engineering of their interaction as a part of actual plant development or life cycle. Switching can be achieved in a variety of ways: by hybridization of two plants (bringing the two genetic components together in the hybrid cell) or by delivery of a message/component from outside the cell (a small molecule activator for a chemical switch; or a virus/bacteria that delivers a more complicated effector molecule to plant cells). Since, at present, the most reliable process is hybridization, we shall give more attention to possible scenarios that involve sexual hybridization as a 'switching step'. In this regard, some considerations will be given to localization of the genetic components on a chromosome relative to each other.

In its most abstract form, the system of a 'germplasm control' has been postulated by Kuvshinov and colleagues (2001), who proposed a two-factor concept based on a 'blocking sequence' linked to the gene of interest and a 'recovering sequence', all assembled within one construct. Action of the blocking sequence leads to cell death, or to impairment of sexual reproduction, while the action of the recovering sequence is induced by externally applied chemical or physical factors, leading to the recovery of the normal plant phenotype. Examples of blocking and recovery sequences can be barnase and barstar genes under control of tissue-specific (sulfhydryl endopeptidase) and inducible (heat shock) promoters, respectively.

Two-component systems utilizing 'chemical switches' for transcriptional control of the gene of interest

The general design of this type of system is similar (see paragraph '*Chemically regulated promoters*'), and requires a gene of interest under control of a promoter that is regulated by a suppressor or an activator. The suppressor/activator is expressed from a separate gene, and its interaction with the promoter of the gene of interest requires the presence of a small molecule, such as tetracycline or a steroid. As a result, the gene can be selectively activated by application of the chemical inducer. Among the limitations of these systems are:

(1) a rather low expression level provided by regulated promoters in the 'on' state;
(2) leakiness of the promoters in the 'off' state;
(3) high costs; and
(4) safety concerns associated with the use of most small chemicals-activators. The PCT application of Bright *et al.* (1994) describes a gene cascade consisting of a gene switch linked to a repressor gene, and a repressible operator linked to a protein capable of disrupting plant development. Growth of the plant can be controlled by applying or withholding a chemical inducer. The system is said to be useful for controlling escape of transgenic plants by making their growth and development dependent on continued application of the inducer.

Other systems that rely on transcriptional control of the gene of interest

Some of the first publications that describe a system for control of gene expression are the patents of Oliver *et al.* (1999). These patents claimed several two-factor processes, including the use of a gene under control of a repressible promoter and a repressor, as well as the use of a gene that can be activated by DNA rearrangement induced by a recombinase. The general goal of the authors was to design a system that would allow restricting expression of a transgene to a particular stage of plant development, to a particular tissue, to particular environmental conditions, or to a particular seed generation.

Another specific goal was to develop a technology that would limit transgene expression (or formation of a viable plant) to the first hybrid seed generation only, thus making it impossible to use part of the harvest for replanting. It is difficult to discuss the merits of such technical solutions since the authors did not publish any results that would confirm the efficiency of the process. The patent publication, however, drew a lot of attention by opponents of plant biotechnology, who dubbed the approach as 'the terminator technology' and declared that its implementation would threaten farmers by making them even more dependent on large seed and agrochemical companies. The regretful result of this political campaign was that Monsanto officially, and several other companies unofficially, took the decision to not pursue the development of this or similar technologies. In our opinion, the principles described in this invention are useful technical concepts that, if implemented, would allow for control of germplasm or transgene movement, which is essential for transgenic plants of classes III or IV (as defined above).

Here, we refer to the work of Schernthaner *et al.* (2003), who described a two-component system for control of seed germination (*Figures 14.4* and *14.5*). The

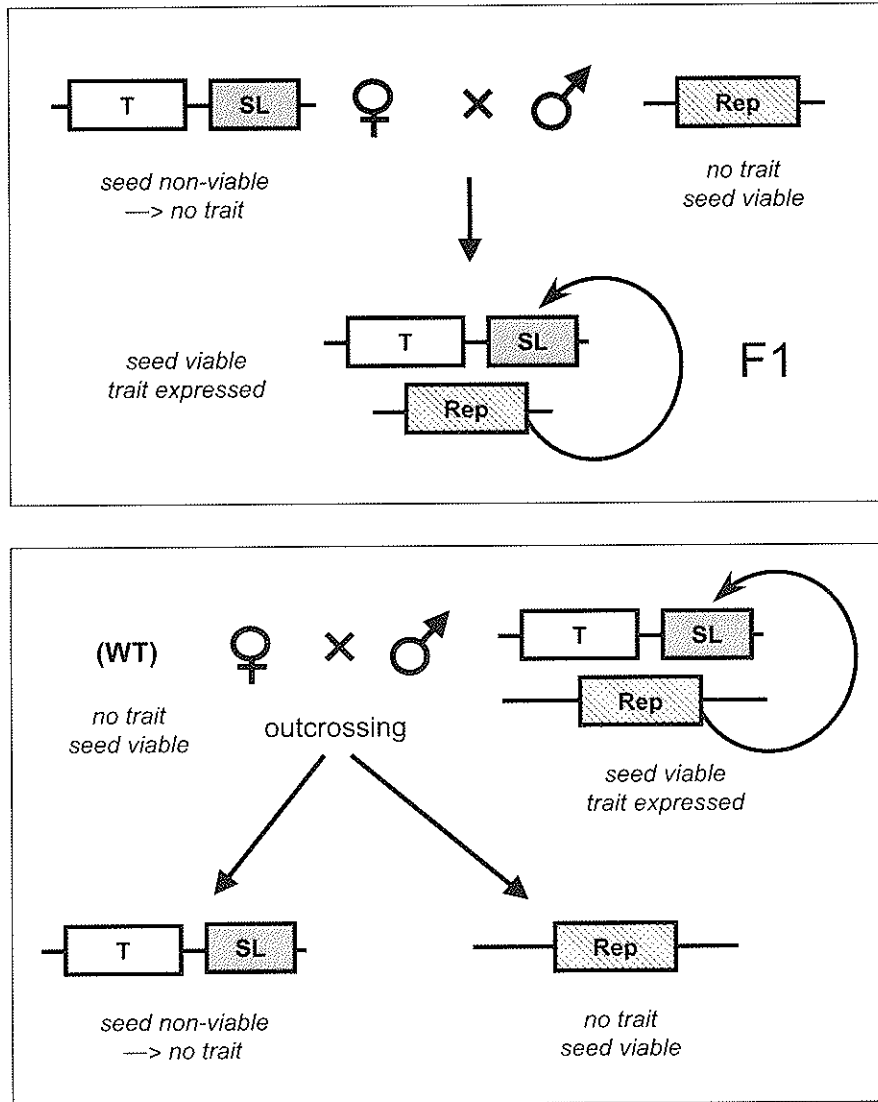
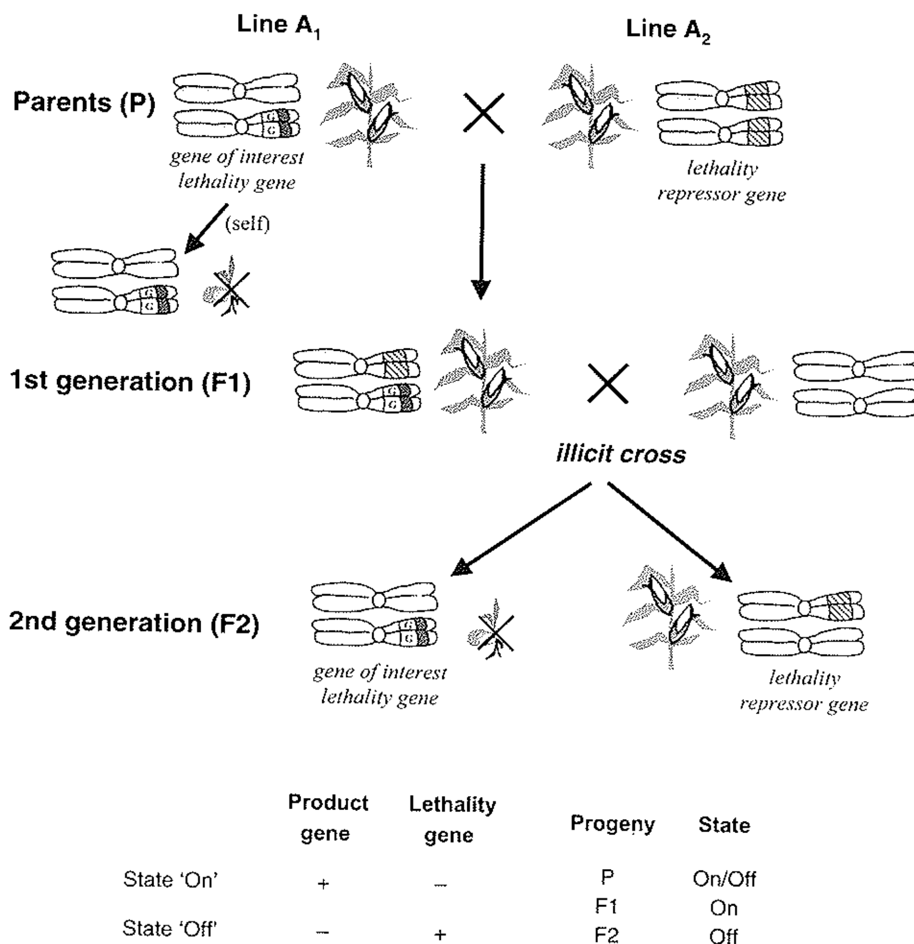


Figure 14.4. Process of controlling transgene spread that relies on linkage between the transgene of interest and a lethality gene that requires the presence of a repressor – a separate genetic element, for plant viability (Schernthaner *et al.*, 2003). A two-component system in which the gene of interest is linked to a lethality gene, which lethality is repressed if a lethality repressor, preferably located on an opposite homologous chromosome in the same locus, is present.

authors proposed a system that relies on a seed-lethal gene (in their particular example, the *Agrobacterium* genes iaaM and iaaH that cause auxin overproduction in embryos and result in seed lethality) and a transcriptional repressor (preferably located on another chromosome) that is required to prevent expression of the seed-lethality gene. In the proposed case, the seed-specific phaseolin promoter used to drive the first lethality gene was engineered to contain a binding site for the bacterial tet repressor. The system requires the presence of both the seed lethality gene (linked



	Product gene	Lethality gene	Progeny	State
State 'On'	+	–	P	On/Off
			F1	On
State 'Off'	–	+	F2	Off

Figure 14.5. Gene flow control and relationship between the transgene of interest and lethality gene in the process proposed by Schernthaner *et al.* (2003). The illicit progeny will, in all cases, separate the lethality gene/gene of interest, on one side, and lethality repressor gene, on the other side (i.e. the two component traits are 'linked in repulsion'), leading to sterility of the progeny that contains the gene of interest. The movement of the lethality repressor, however, is not controlled.

to the gene of interest) and the lethality suppressor. Any illicit cross that leads to transmission of only one component will deactivate repression and lead to elimination of the plants with the gene of interest from the population. The most interesting version of the system, as discussed by the authors, was when the two components were positioned at the same locus on two homologous parental chromosomes (components 'linked in repulsion'). This configuration will lead to complete elimination of all progenies carrying the gene of interest upon cross-pollination. Unfortunately, the authors did not explain how such a heterozygous population of plants could be effectively maintained (upon selfing, such plants would segregate both desired heterozygotes, as well as viable but undesired homozygotes carrying the repressor gene). In addition, their scenario had a serious drawback, the failure to control the second transgene component, i.e. the repressor gene.

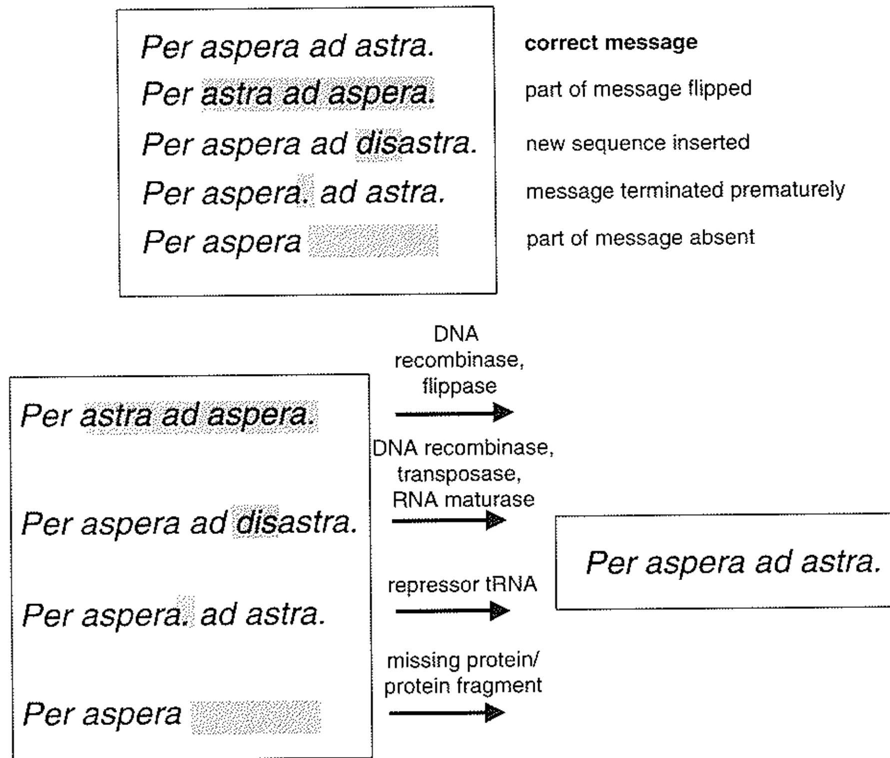


Figure 14.6. Encrypted information scenarios and possible biological mechanisms leading to unencryption of genetic information. The Latin message ('per aspera ad astra') translates as 'over thorns to stars'. Different corrupted versions of the message (A), as well as different 'biological tools' that can be used to correct a similarly corrupted/encrypted genetic message (B), are listed.

A different type of approach based on multi-component systems, but not relying on transcriptional control of the gene of interest, consists of using genes of interest or constructs with altered or 'scrambled' coding instructions. As a result, any escaping genetic material would be completely non-functional if incorporated into an organism that does not possess the appropriate decoding or 'message descrambling' capabilities. This approach can be implemented using multiple genetic elements, and is illustrated (*Figure 14.6*) by altering a simple written message in different ways. By using existing biological processes, one can design 'unencryption' mechanisms at a transcription level (site-specific recombinases, transposases), RNA processing level (ribozymes, maturase), translation level (tRNA, ribozymes, acetyl synthases), or post-translational level (intein, specific targeting to subcellular compartment).

Reconstitution of functionality via site-specific recombination

A number of site-specific recombination systems have been tested and shown to work in plants with high fidelity (for a review, see Ow, 2002). Site-specific recombination results in insertions/excisions, inversions or translocations, depending on the

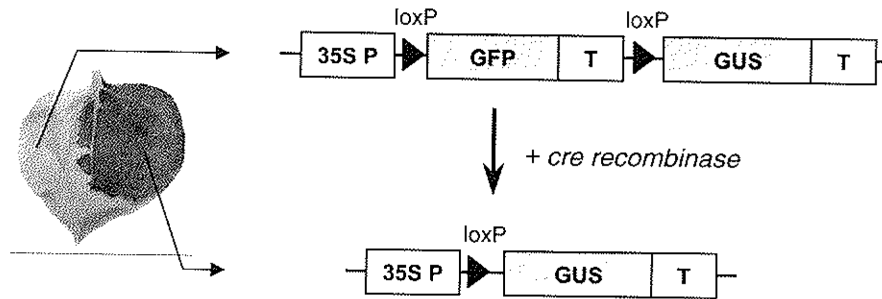


Figure 14.7. Recombinase-mediated unencryption of gene expression through site-specific removal of an intervening DNA insert. Removal of an insert in a stably transformed DNA construct, by transient expression of cre recombinase using *Agrobacterium*-mediated delivery, results in expression of the gene of interest in the infiltrated area (in this case GUS).

relative orientation of the recombination sites on a chromosome, or their position on different chromosomes. It is therefore possible to design non-functional constructs by addition of disruptive sequences flanked by recombination sites, or by 'flipping' part of the construct between two recombination sites, and to reactivate these constructs by expression of the appropriate recombinase.

Recombination can be induced either by hybridization with a plant expressing recombinase, or by transcriptional activation of a recombinase gene using a regulated promoter. For example, Hoa and colleagues (2002) showed that an inactive GUS reporter gene in rice plants could be reactivated in the hybrid progeny by expression of Cre recombinase and removal of a blocking DNA insert (for our similar experiments, see *Figure 14.7*). An example of activation of a construct by recombinase-mediated DNA 'flipping' (work done in our lab) is shown in *Figure 14.8*. So far, a large proportion of the work done in plants has used site-specific recombination to delete parts of an integrated vector, either to remove a selectable marker (Sugita *et al.*, 2000; Corneille *et al.*, 2001; Zuo *et al.*, 2001; Hoa *et al.*, 2002) or to resolve complex integration patterns (Srivastava *et al.*, 1999). We expect that in the future, site-specific recombination will be used more often to perform tasks of higher complexity.

Ribozymes and reconstitution of functionality at the RNA level through inter- or intramolecular recombination

Over several years, intermolecular RNA recombination catalysed by engineered *trans*-splicing ribozymes has been actively explored. Ribozymes (Mikheeva and Jarrell, 1996; Ayre *et al.*, 2002) have been engineered mainly from Group I and Group II introns (for a review see Saldanha *et al.*, 1993; Cech, 1995). Recently, spliceosome-mediated *trans*-splicing of mRNA was shown also to be possible (Puttaraju *et al.*, 1999; Garcia-Blanco, 2003). Use of RNA *trans*-splicing was tested in plant and animal systems, primarily to mediate repair of defective mRNAs, or to confer toxicity and eliminate cells that had acquired undesired RNAs, such as viral RNAs (Sullenger and Cech, 1993, 1994; Perriman *et al.*, 1995; Lan *et al.*, 1998; Ayre *et al.*, 1999). This specific use was dictated by the relatively low efficiency of *trans*-splicing. Thus, use of ribozyme-mediated *trans*-splicing is limited to applications

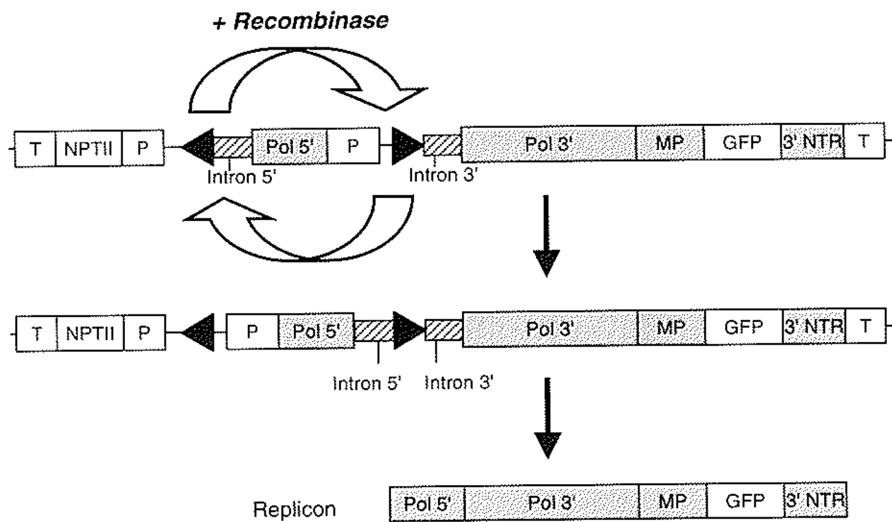


Figure 14.8. Recombinase-mediated unencryption of a viral amplicon through site-specific flipping of the part of gene encoding viral polymerase. Recombinase-mediated flipping of a DNA sequence results in formation of an active RNA amplicon capable of cell–cell movement and transgene expression.

where a very low amount of *trans*-spliced RNA is sufficient to obtain the desired phenotype. The process is, however, highly specific, and can be tailored to target almost any RNA, and as such, ribozymes represent a very interesting tool for the genetic engineer's 'toolbox'.

We have demonstrated in our laboratory that *trans*-splicing of two separate inactive RNA amplicon fragments could lead to formation of a functional replicon. The replicon is able to move to neighbouring cells as well as systemically, thus performing the roles of an 'amplifier' inside the cell and of a 'secondary messenger' by spreading to other cells (Ivanov *et al.*, unpublished results; Klimyuk *et al.*, 2002). In yeast, reconstitution of a functional RNA was obtained by intramolecular recombination using a heterologous (mitochondrial) maturase, which normally catalyses splicing of two yeast mitochondrial introns (Banroques *et al.*, 1986, 1987). Despite the presence of maturase encoding genes in plants (Wahleithner *et al.*, 1990; du

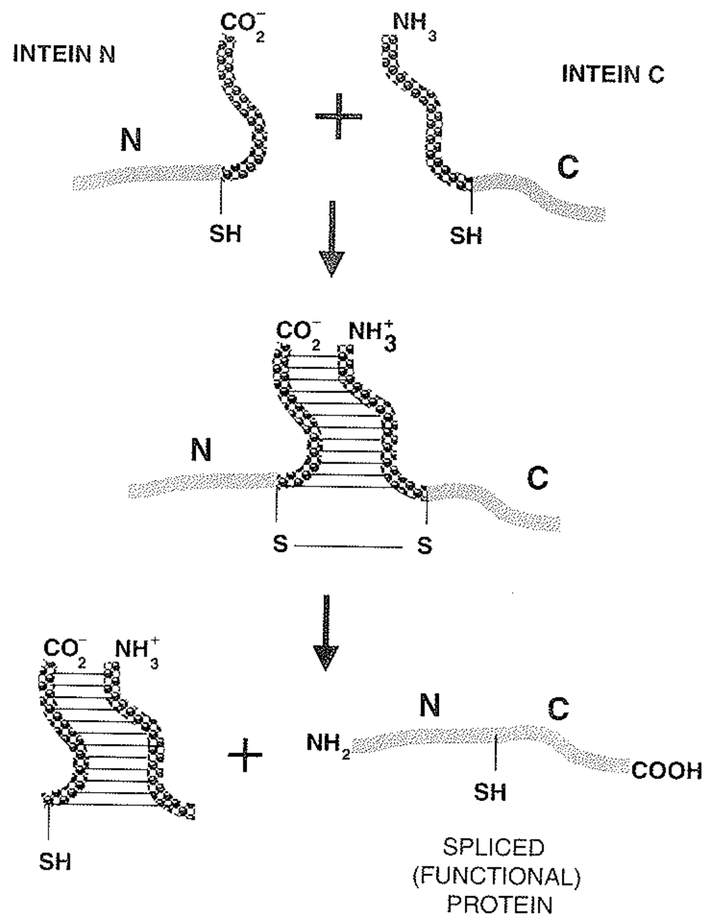


Figure 14.9. Scheme illustrating the principle of intein-mediated protein *trans*-splicing. Expression of the two intein fusions in the same cell will lead to an enzymatic reaction resulting in highly specific and efficient polypeptide splicing and formation of a functional protein.

Jardin *et al.*, 1994; Vogel *et al.*, 1997; Mohr and Lambowitz, 2003), we are not aware of similar work in plants.

Trans-*splicing inteins: reconstitution of a functional protein from non-functional fragments*

Inteins are protein fragments that are embedded within precursor proteins, and which are able to excise from the precursor by a post-translational processing event called protein splicing (Perler, 1998; Perler and Adam, 2000; Gogarten *et al.*, 2002). Inteins are able to catalyse not only *cis*-splicing reactions, but also *trans*-splicing events between separate protein fragments (Wu *et al.*, 1998a,b; Liu and Yang, 2003). Therefore, inteins can be used to reconstitute a functional protein from inactive fragments. Reconstitution of a functional protein in plants by intein-mediated *trans*-splicing was demonstrated recently (Yang *et al.*, 2003; and work done in our lab, see *Figures 14.9* and *14.10*).

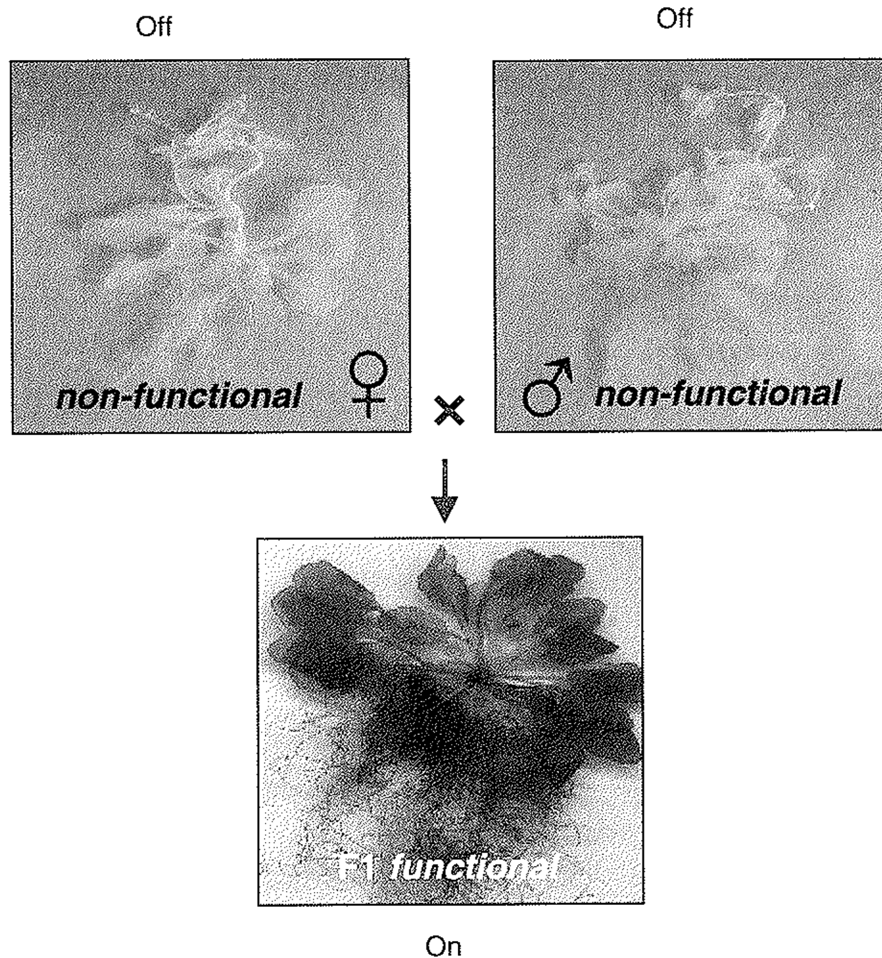


Figure 14.10. Successful assembly of a functional protein assisted by intein-mediated *trans*-splicing in a hybrid progeny that inherited two genes, each encoding non-functional fragments. Note the absence of enzymatic activity in the two parental lines, each expressing different non-functional GUS fragments as intein fusions; and the *trans*-splicing and restoration of full activity in the hybrid that inherited both complementing GUS–intein fragments.

The ability of intein fragments to catalyse *trans*-splicing events can be used for building multi-component systems characterized by a highly reduced possibility of transgene escape. For example, a transgenic plant containing both fragments of an intein-split gene will express a fully functional protein. However, only part of the progeny inheriting both transgenes will also express the functional protein. If the gene fragments are inserted at the same locus, but each fragment on a separate (homologous) chromosome, none of the progeny of an 'illicit' cross with a non-transformed plant will inherit both copies and express the transgene (*Figures 14.11* and *14.12*).

In an alternative configuration, the two coding fragments can be transformed into different cell compartments, such as the nucleus and the chloroplast (Chin *et al.*,

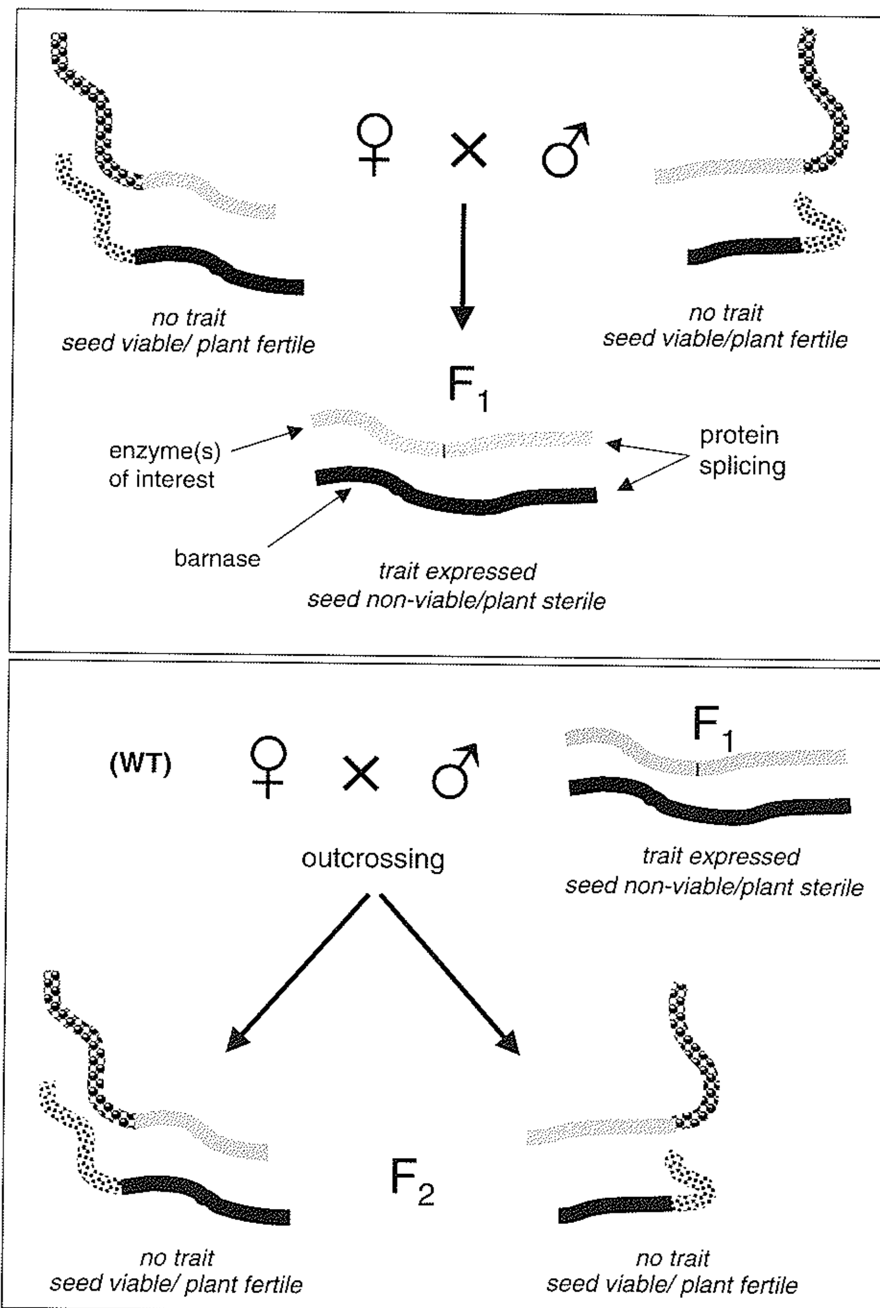
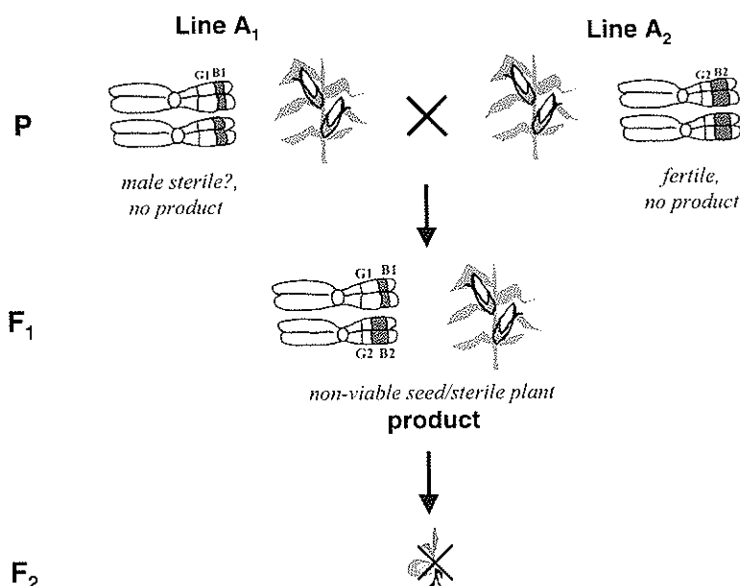


Figure 14.11. A general scheme describing a trait lock technology that is in essence a dual switch that links expression of the transgene of interest to the expression of a lethality gene, thus rendering a 'production seed' incapable of further sexual reproduction. A simple principle that links two processes that are both activated as a result of hybrid seed formation. The developmental block can be achieved in many different ways; it may arrest development beyond seed formation and prevent seed germination, root formation, normal plant development, fertility of the progeny, etc.



	Product gene	Lethality gene
State 'On'	+	+
State 'Off'	–	–

Progeny	State
P	Off
F1	On
F2	Off/On

Figure 14.12. Trait lock technology: gene flow control and relationship between expression of a transgene of interest and of a lethality gene. The system allows for clear separation of breeding and material multiplication steps versus the final product seed production step, i.e. the 'growth phase' and the 'production phase' are fully separated. Note that the system provides benefits other than biosafety: the product does not accumulate in the parental lines, thus there is no adverse effect on growth/development or yield (agronomic parameters) prior to or during production; the produced seed cannot be used for re-planting, thus providing for a better control over the process.

2003). Since plastid genes are inherited uniparentally (maternally) in most species, the frequency of escape of a functional trait by out-crossing is lowered by several orders of magnitude. By additionally equipping the system with a male sterility gene, one could very effectively suppress the ability of the transgene to escape. One limitation of such a system is the current lack of plastid transformation methods for species other than tobacco.

Intein-assisted *trans*-splicing is a potentially very versatile tool, since many different inteins have been described, and because several sites can be used within one protein. Therefore, inteins can be used by the genetic engineer to design complex systems containing multiple independent components. In our laboratory, we have used intein-based *trans*-splicing to split and reconstitute several useful proteins, including non-selectable (GUS) and selectable (acetolactate synthase) markers, a toxic protein (barnase) and a recombinase (integrase).

It has also been shown that reconstitution of protein function can sometimes be achieved by alternative means. In some cases, simple co-expression of non-

overlapping peptide fragments can be sufficient for function (Sancho and Fersht, 1992; Serrano *et al.*, 1993; Kwon *et al.*, 1995; van der Ent *et al.*, 1999). In other cases, reconstitution of protein function was obtained by introducing affinities (e.g. use of leucine zippers and the like) that did not involve peptide bond formation (Karimova *et al.*, 1998; Pelletier *et al.*, 1998, 1999). Unassisted assembly of barnase (a highly toxic RNAse) from non-overlapping fragments was demonstrated a decade ago (Sancho and Fersht, 1992; Serrano *et al.*, 1993), and this feature was recently incorporated into a hybrid seed system to confer male sterility (Burgess *et al.*, 2002).

Unencryption utilizing alternative coding instructions

Theoretically, an ideal solution for a safe 'transgene operating system' would be to express the transgene using coding instructions different from those operated by nature. The system would require several components: 1) a message that is written using new coding instructions, and 2) a toll that would allow the message to be decoded. The advantage of this approach is that the transgene would not be expressed in a plant lacking the new coding instructions. In practice, using a modified coding system in an otherwise normal cell would lead to chaos. However, such a system could still be implemented by a temporary 'violation' of normal coding instructions, for example to activate a genetic switch or to trigger a cascade.

Similar systems (though with fewer modifications than in the system proposed above) have, in fact, been known in genetics for decades. Most of them are based on non-sense mutation suppressor tRNAs that lead to incorporation of amino acids, instead of terminating protein translation, when reading stop codons (Murgola, 1985). In its simplest form, the system would require a transgene containing one or more non-sense mutations, and a corresponding suppressor tRNA gene that could be activated when needed (*Figure 14.13*). Similar schemes have been proposed

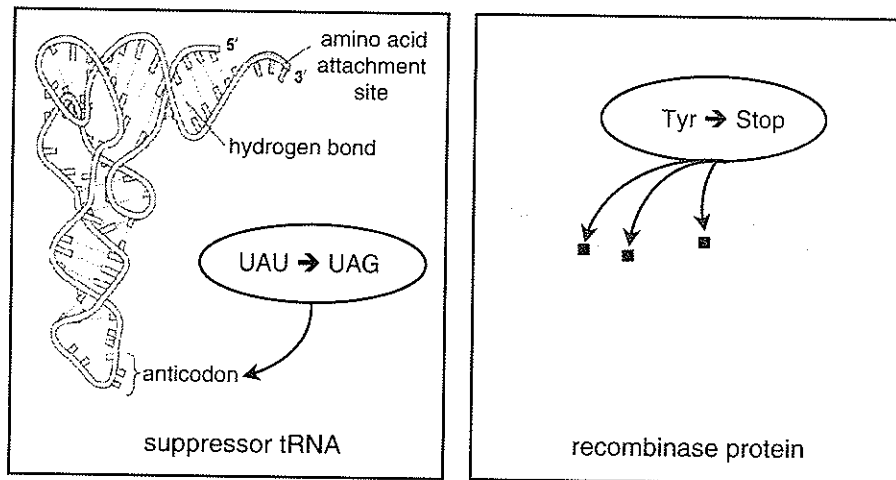


Figure 14.13. A general scheme illustrating an unencryption approach that is based on suppression of non-sense mutation by expression of suppressor tRNA. The system is obviously useful for highly specific switching, such as activation of a recombinase that will, in turn, trigger a cascade of events; in most cases, subsequent 'amplification' of the primary signal will be necessary.

for *trans*-activation in plants (Carneiro *et al.*, 1993; Betzner *et al.*, 1997; Choisne *et al.*, 1997). Carneiro and colleagues (1993) demonstrated that a mutant GUS gene carrying three stop codons could be expressed transiently in tobacco protoplasts after co-electroporation with suppressor tRNA genes. In two other experiments, a mutant GUS gene was expressed in *Arabidopsis thaliana* (Betzner *et al.*, 1997) and tobacco (Choisne *et al.*, 1997) after hybridization with transgenic plants carrying suppressor tRNA genes.

Relative position of genetic components on a chromosome

Several authors dealt with the issue of the relative position of multiple transgenes on a chromosome (Fabijanski *et al.*, 1999; Schernthaner *et al.*, 2003). This is an important issue that will affect the segregation patterns of transgenes in the progenies of either intentional or illicit crosses. If a transgene or trait of interest (T) is combined with a seed lethality gene (SL) to prevent escape of the transgene by outcrossing (as described above in the work of Schernthaner *et al.*, 2003), it is obvious that the transgene and repressor have to be present on the same chromosome, on the same locus. Engineering such a linkage is usually not a problem, and is achieved by introducing both genes as one DNA construct. Correct positioning and introduction of the other component of the lethality system, such as a repressor (Rep) of seed lethality (*Figures 14.4* and *14.5*), is a much less straightforward task, and it can be designed in a number of ways requiring different genetic manipulations. One of the most powerful solutions is when both components of the system (transgene of interest/lethality gene and lethality repressor) are located at the same locus, but on different homologous chromosomes. The two components, alleles, are then 'linked in repulsion', i.e. during meiosis, each gamete will inherit either one or the other, but not both. This application for transgene control has been proposed by Fabijanski *et al.* (1999), who suggested such genetic positioning in designing a two-component system that would transmit only one of the components to sexual progenies. Specific ways for putting different genes at the same locus on homologous chromosomes have not been discussed. One of the approaches that we use in our work is first to transform both components as one pro-locus, and then, as a second step, to remove alternative parts of the pro-locus by transposition or site-specific recombination, resulting in two alleles each having only one of the components left (*Figure 14.3*).

Delivery and transient expression of DNA into a plant cell

Agrobacterium-mediated gene delivery into plant cells is a very efficient process (Kapila *et al.*, 1997; Vaquero *et al.*, 1999; Voinnet *et al.*, 2003). The relatively low efficiency of recovery of stable transformants is attributed to limiting events taking place after T-DNA delivery by *Agrobacterium* to the plant cell, rather than by the efficiency of T-DNA transfer (Weld *et al.*, 2001). In experiments performed in our lab, we have found that, by co-infiltrating *Nicotiana benthamiana* leaves with a mixture of two *Agrobacterium* cultures carrying different expressible markers, co-expression in transfected plant cells was close to 100% (Marillonnet *et al.*, 2004). Therefore, *Agrobacterium*-mediated DNA delivery represents a very efficient and easy to use technique for transient expression in plants that can also be used as a

genetic switch. In our experiments, *Agrobacterium*-mediated transfer is used to activate a DNA copy of an RNA viral vector encrypted on a chromosome, by delivery of a construct expressing recombinase (*Figure 14.8*).

Delivery of proteins into a plant cell

Some pathogenic bacteria have the ability to deliver proteins to plant cells using specialized protein translocation systems (for reviews see: Ream, 1998; Collmer *et al.*, 2000). *Pseudomonas* and *Xanthomonas*, for example, use a type III secretion system for delivery of bacterial proteins across the bacterial and eukaryotic plasma membranes (Casper-Lindley *et al.*, 2002; Szurek *et al.*, 2002). Transfer of bacterial proteins to plant cells elicits defense responses and/or causes disease. Until now, most studies on secretion systems have focused on the analysis of pathogenicity mechanisms. It is, however, obvious that protein delivery could be developed as a useful tool for biotechnology applications. As a step in that direction, it was shown that the protein delivery system of *Erwinia* could be transferred to non-pathogenic bacteria such as *E. coli*, and retain full functionality (Ham *et al.*, 1998).

Agrobacterium is also capable of transferring protein to plant cells. However, in this case, the transfer is more complex and involves transfer of both DNA and proteins. Furthermore, it is known that Agrobacteria use at least a second translocation system for translocation of VirE, type IV secretion system, which also functions in the absence of active DNA transfer. In a pioneering work (Vergunst *et al.*, 2000), bacterium-mediated protein transfer was used to deliver recombinase to plant cells. Delivery was detected when Cre recombinase was expressed as an NH_2-terminal fusion to either VirE2 or VirF. Delivery was detected using a sensitive selection assay based on kanamycin resistance, which occurred as a result of a cre-mediated recombination event. Although detectable, efficiency of the process was low. Such a system could therefore be used only for applications where rare recombination events can be screened with some type of selection.

It has been demonstrated that proteins having so-called membrane-translocation sequences (MTSs), which are positively charged peptide extensions, are capable of entering cells through the plasma membrane. Translocation of such proteins occurs in an energy-independent way, probably by exploiting the asymmetry of the plasma membrane, in which the internal lipid monolayer facing the cytoplasm contains anionic phospholipids (Buckland and Wilton, 2000). An MTS might be a simple synthetic amino acid repeat, e.g. a repeat of arginine residues (Matsushita *et al.*, 2001), or short arginine/lysine-rich peptides (Mi *et al.*, 2000), or be derived from the MTSs of viral and cellular proteins (Wender *et al.*, 2000; Futaki *et al.*, 2001). It was shown that all these positively charged MTSs are able to penetrate cells alone or as fusions with other proteins, e.g. GFP (Han *et al.*, 2000), Cre recombinase (Peitz *et al.*, 2002), or when chemically linked to antibodies (Zhao *et al.*, 2001). This approach is widely used in peptide-mediated therapeutic delivery systems (Schwartz and Zhang, 2000; Gratton *et al.*, 2003).

It remains to be seen if protein delivery can be optimized to such an extent as to be useful for broad applications in plant biotechnology; for example, as switches. With the low efficiencies obtained with the protocols described until now, only applications with a powerful 'amplification' step after protein delivery may be considered.

One of the applications in development in our laboratory consists of activating replication of a viral vector present in an inactive form in a transgenic plant by using a switch based on delivery of either a DNA or protein molecule.

Small molecules as chemical switches

The ability to 'turn on' gene expression (and, more generally, a biological process) using a small molecule has been a topic of perennial interest in many laboratories. Large agrochemical companies were obviously hopeful that such a chemical trigger would allow them to use their expertise in the chemistry of small molecules (such as pesticides and growth regulators) to develop biotechnology processes that are more controlled. Several systems have been developed, including systems that use the antibiotic tetracycline (Gatz and Quail, 1988; Gatz *et al.*, 1992; Weinmann *et al.*, 1994; Gatz, 1997), steroids (Aoyama and Chua, 1997; McNellis *et al.*, 1998), copper (Mett *et al.*, 1993), ethanol (Caddick *et al.*, 1997; Salter *et al.*, 1998; Roslan *et al.*, 2001), acetaldehyde (Junker *et al.*, 2003), the insecticide methoxyfenozide (Padidam *et al.*, 2003). Unfortunately, most of the systems available at present do not allow a tight control under non-induced conditions. Moreover, except perhaps ethanol, the use of many chemical inducers, such as antibiotics and steroids, is not desirable for large-scale applications in an open field. These chemicals might, however, have applications in contained environments such as growth chambers or greenhouses.

OTHER COMPONENTS OF A 'TRANSGENE OPERATING SYSTEM'

In all preceding descriptions of the relevant technologies and components, we often referred to a number of standard genetic tools, such as selection markers, counter-selectable markers (toxic enzymes, lethality molecules), site-specific recombinases, *trans*-splicing inteins, ribozymes, constitutive, regulated and tissue-specific promoters, etc. Molecular genetics provides a variety of such elements, but usually, use of specific elements optimal for the particular task is necessary. For example, the resistance genes to the broad-spectrum herbicides glyphosate, phosphinothricins, imidazolinones, and sulfonylureas and the respective herbicides have all been the subject of extensive toxicological studies, and have been registered for field use. It has to be remembered that, for our purpose, the resistance gene is used for a specific technology solution, rather than as a herbicide resistance gene. These two roles may actually be in conflict for certain scenarios. Furthermore, the owners of some genes may object to a specific use, as these genes may have been developed for a different purpose, with a larger market target in mind. Intellectual property issues, and the resulting complications, add additional layers of complexity for the development of the best overall process, but analysis of these issues is outside the scope of this review.

Apart from a proven safety record and 'freedom-to-operate', there are numerous technical reasons for choosing some tools over others for a specific process. Essential elements are efficiency, robustness, and reliability of performance. Most of the tools listed below in our proposed standard 'toolbox' have been extensively used in many genetic experiments, and have a stellar record of performance *in vitro*. It is, therefore, natural to extend their use beyond a Petri dish. Another reason may be a

proven record of performance in plants. Here, we are often confronted with two conflicting considerations. In many cases, we prefer tools that do not cross-communicate with other plant processes, while in other cases the opposite is required. For example, a promoter used for controlled expression in a transgenic plant should not also be up- or down-regulated by stress-related or physiological processes. Also, use of transposase as a switch in maize would not be a preferred choice due to fears that the engineered host may have, or may acquire, an active transposase by hybridization with other lines. As a result, we end up borrowing a tool from a bacterial genome, or another unrelated living organism. In the future, we will probably develop artificial tools. The added attraction would be a greater choice of specific reactions, and the ability to use tools that do not communicate with plants or other organisms (such as bacteria). In both cases, use of 'xenogenic' elements will require additional studies to demonstrate that they are safe.

Below is a brief review of the genes, genetic elements, and systems that are deemed most useful as parts of a safe transgene operating system.

Selectable markers

Genes conferring a commercial level of resistance to broad-spectrum herbicides, such as glyphosate, phosphinothricin, imidazolinones, sulfonylureas, and bromoxynil, have been engineered into plants, and herbicide-resistant GM plants developed and successfully commercialized. All mentioned resistance genes have been extensively studied with regard to safety and efficiency for weed control. All of them can also be used as selective markers for R&D, as well as production purposes. Use of a herbicide as a 'technology herbicide' (for example, to select for male sterile progeny only), rather than as a 'weed herbicide', may sometimes be conflicting; luckily, there is a choice of other genes and matching chemicals to select from. Numerous antibiotic resistance markers used in first-generation GM plants (Rothstein *et al.*, 1987; Damm *et al.*, 1989; Meijer *et al.*, 1991; Walters *et al.*, 1992; Perlak *et al.*, 1993), such as kanamycin or hygromycin resistance from bacterial plasmids (Gritz and Davies, 1983; Thompson and Gray, 1983; Mazodier *et al.*, 1985), have all been declared 'undesired'. Therefore, their use in the future will be limited to early R&D stages.

Counter-selectable markers and lethality proteins

Suppressing unwanted genotypes or killing specific cells/tissues are both important procedures that have been used extensively in genetic studies (Perera *et al.*, 1993; Goldman *et al.*, 1994; O'Keefe *et al.*, 1994; Day *et al.*, 1995; Koprek *et al.*, 1999; Tsugeki and Fedoroff, 1999). However, except for the use of barnase for cell ablation, these tools are still not commonly used in plant biotechnology. Barnase is a highly toxic RNAse that can kill a cell with just a few molecules. It was shown to be functional in plants, and to allow cell ablation if placed under control of an appropriate tissue-specific promoter. Its expression from a stigma-specific promoter was shown to produce female-sterile plants (Goldman *et al.*, 1994), while expression from a tapetum-specific promoter resulted in male sterility (Denis *et al.*, 1993). Interestingly, barnase was also found to work when expressed as two peptide

fragments, and activity did not even require assembly of the fragments into a single molecule. This feature was used to design a hybrid seed system in which tissue-specific barnase expression was achieved by expression of both fragments from promoters with different but overlapping patterns of expression (Burgess *et al.*, 2002). Because the fragments are non-functional when expressed separately (in parental lines), the system avoids problems of general toxicity encountered when a complete gene is used, even using a tightly regulated pollen-specific promoter.

One of the counter-selectable markers most commonly used in plants is cytosine deaminase (Perera *et al.*, 1993). One limitation for the use of this gene is the need for a chemical that has not been registered for outdoor use. An alternative counter-selectable marker that is more amenable for large-scale use in field conditions is bacterial cytochrome P450SU1 that converts a sulfonylurea pro-herbicide (R7402) into a cytotoxic metabolite (O'Keefe *et al.*, 1994; Koprek *et al.*, 1999). Examples of 'milder' counter-selectable markers for plants are *Agrobacterium* genes involved in auxin and cytokinin biosynthesis. Expression of these genes in plants leads to auxin and cytokinin overproduction, resulting in developmental abnormalities and an inability for the plant to pass specific developmental phases. One of these genes, the isopentyl transferase gene, has been used extensively, in particular for the production of transgenic plants without a selectable marker (Ebinuma *et al.*, 1997; Kunkel *et al.*, 1999; Sugita *et al.*, 2000; Endo *et al.*, 2002; Sa *et al.*, 2002). Two other genes, the iaaM and iaaH genes, that are involved in indole-3-acetic acid production, have been used as part of a recently described seed lethality system (Schernthaner *et al.*, 2003).

DNA recombinases

Several site-specific recombinases have been used extensively in genetics, and were also found to perform well in plants: cre recombinase (Vergunst *et al.*, 2000; Corneille *et al.*, 2001; Srivastava and Ow, 2001; Mlynarova and Nap, 2003), flp recombinase (Lyznik *et al.*, 1996; Luo and Kausch, 2002), R recombinase from *Z. rouxii* (Onouchi *et al.*, 1995; Sugita *et al.*, 2000), and ϕC31 integrase (Zubko *et al.*, 2000). Although, so far, most research in plants involved cre or flp recombinases, the recombination events catalysed by integrase are non-reversible; therefore, this enzyme may be more appropriate for certain purposes. Site-specific DNA recombination attracts a lot of interest because of the potential use for targeted integration of transgenes at a predetermined locus on a chromosome. Such a transgene operating system, based on the use of a predetermined 'transgene locus', offers numerous advantages, as compared to present transformation methods that result in random integration of the transgene: firstly, the repeated use of a known integration locus permits a more predictable control of transgene expression (and a straightforward event selection and development); secondly, line conversion could be made faster, cheaper, and without a 'linkage drag' (i.e. without the transfer of undesired donor DNA material other than transgene *per se*). It was demonstrated that the specificity of cre recombinase for recombination sequences could be effectively manipulated by directed evolution (Santoro and Schultz, 2002). This means that, in theory, different recombinases can be developed to work independently, and to be used simultaneously for an engineering process within the same host.

Trans-*splicing inteins*

The field of intein-based *trans*-splicing is very young, with *trans*-splicing of heterologous proteins having been reported just 5 years ago (Shingledecker *et al.*, 1998; Wu *et al.*, 1998a,b). As of today, two intein systems have been shown to work effectively in plants: the naturally split DnaE intein (Chin *et al.*, 2003), and the artificially split DnaB intein (Wu *et al.*, 1998b), both coming from *Synochocystis* sp. Because of the high degree of reconstitution provided by inteins, we expect that switches based on inteins will become popular in the future; in fact, much of our own research is focused on intein-mediated *trans*-splicing. For example, expression of an active protein of interest by expression of two protein fragments from two different promoters with overlapping activity (or a combination of inducible and tissue-specific promoters) would provide a significantly tighter control of expression.

Amplicons

Although not specifically a part of any safety system *per se*, amplicons of viral origin are often used to reach a high level of expression, or to design a process with a short expression time and a high throughput. They are also useful when the primary switch is imperfect and shows low penetration. This may be the case when certain recombinases are used to activate replication by flipping or deleting a DNA fragment on a plant chromosome (*Figures 14.7* and *14.8*). Since recombination may occur in only some of the cells of the host, there is a need to 'amplify' the recombined product within the cell and 'export' it to neighbouring cells. In a leaf system, an ideal candidate for such activity is an RNA amplicon derived from Tobacco Mosaic Virus (TMV), engineered to express a function of interest. The vector has the ability to spread to nearby cells, and, depending on the design, the ability to move systemically throughout the whole plant (Donson *et al.*, 1991; Kumagai *et al.*, 1993, 2000). In addition to TMV-based vectors, several other vectors derived from RNA and DNA viruses have been shown to perform in plants (Stanley, 1993; Porta and Lomonossoff, 1996; Mallory *et al.*, 2002; Mor *et al.*, 2003). Plant viral vectors have been used not only for overexpression of a gene of interest, but also for functional genomic studies using gene silencing (Kumagai *et al.*, 1995; Baulcombe, 1999; Fitzmaurice *et al.*, 2002).

Constitutive promoters

The most commonly used heterologous DNA sequence in commercial transgenic plants is the Cauliflower Mosaic Virus 35S promoter (Franck *et al.*, 1980). It provides very strong constitutive expression, and has been used in transformants that have performed reliably for multiple generations, in multiple varieties and crops. The Arabidopsis actin promoter *ACT2* (An *et al.*, 1996) is a comparatively weaker promoter, but is a useful promoter for expression in vegetative tissues. The most frequently used constitutive promoters for monocotyledonous plants are the promoters of the maize ubiquitin 1 (Christensen and Quail, 1996), and rice actin 1 (McElroy *et al.*, 1990) genes. Numerous alternatives are available, but the properties of most of these have often not been researched beyond laboratory scale.

Tissue-specific promoters

Few tissue-specific promoters have been used commercially in GM plants. Notable exceptions are the tissue-specific promoters that are used to engineer male sterility in the hybrid canola seed system developed and commercialized by Bayer Crop Science (WO9325695; WO9626283), and the promoters used to delay fruit ripening (Gray *et al.*, 1992). Cloning (and patenting) tissue-specific promoters has been a favourite occupation of many large and small companies, as well as academia, and those trying to make an informed choice have our full sympathy.

Chemically regulated promoters

There are numerous systems that allow regulation of gene expression by application of a small molecule. Examples of such systems include: a tetracycline-inducible system (Gatz and Quail, 1988; Gatz *et al.*, 1992; Weinmann *et al.*, 1994; Gatz, 1997), a copper-inducible system (Mett *et al.*, 1993), steroid-inducible systems (Aoyama and Chua, 1997; McNellis *et al.*, 1998), an ethanol- (Caddick *et al.*, 1997; Salter *et al.*, 1998; Roslan *et al.*, 2001) or acetaldehyde-inducible (Junker *et al.*, 2003) system, and an insecticide methoxyfenozide-inducible system (Padidam *et al.*, 2003). One system was described that combines several of the systems mentioned above. It consists of a chimaeric promoter that can be switched on by the glucocorticoid dexamethasone, and switched off by tetracycline (Bohner *et al.*, 1999). For a latest review on chemically inducible systems, see Padidam (2003).

The systems described above are of significant interest for applications requiring controlled transgene expression, but unfortunately they do not allow tight control of expression patterns, as the inducing agents (copper) or their analogues, e.g. brassinosteroids in the case of steroid-controllable systems, can be present in plant tissues at levels sufficient to cause some residual level of expression. Additionally, the use of some chemical inducers, such as antibiotics and steroids, is not desirable for large-scale field applications. Therefore, except possibly the ethanol-inducible system, none of these systems is perfect for large-scale commercial applications. The ethanol switch proposed early on by Caddick and colleagues (1997) requires a cheap and safe chemical, and has recently been improved, and could perhaps satisfy the criteria required for commercial use of a chemical gene switch.

Control of bacterial or viral recombination

BACTERIAL RECOMBINATION

It is theoretically possible that a transgene might be transferred from a GM plant to a microorganism or a virus. In laboratory simulations, it was shown that plant DNA can, in fact, be taken up by bacteria and integrated into the bacterial genome by homologous recombination. As a result, antibiotic resistance genes (for example) could be transferred to bacteria and be expressed under particular conditions (Kay *et al.*, 2002; de Vries *et al.*, 2003). It was also shown in the same work that, for recombination to take place, a significant degree of homology was necessary between the transgene and/or flanking sequences and the bacterial genome. The

Acinetobacter sp. strain BD413 was equipped beforehand with DNA sequences homologous to the recombinant DNA present in the plant genome. The authors concluded that, to effectively mitigate the risk of recombination, the constructs of interest should be engineered to avoid the presence of sequences homologous to DNA of known plant-associated bacteria.

A second level of defence consists of incorporating eukaryotic introns into transgenes to prevent expression of the transgenes in prokaryotic cells (Vancanneyt, 1990). This approach is very useful to facilitate the engineering steps that are performed in bacteria, to prevent expression of some of the construct components that might be toxic. For example, it was shown that plasmid engineering of constructs containing genes such as viral replicase (Johansen, 1996; Yang *et al.*, 1998; Lopez-Moya and Garcia, 2000), recombinase (Mlynarova and Nap, 2003), or barnase (Hanson *et al.*, 1999) was made much easier if the genes were modified to contain introns. In addition, incorporating introns into transgenes of either bacterial or eukaryotic origin often results in higher expression levels in plants (Mascarenhas *et al.*, 1990; Bourdon *et al.*, 2001; Rose, 2002). Plants transformed with intron-containing cre recombinase were also found to be less likely to exhibit reduced fertility (Mlynarova and Nap, 2003). One final advantage of using intron sequences is to allow removal of unwanted sequences, such as recombination sites, by splicing. This feature was used in our work for 'in planta' engineering of TMV-based viral replicons (Marillonnet *et al.*, 2004).

VIRAL RECOMBINATION

Viral recombination *in planta* is a well-known phenomenon, and reconstruction of a wild-type virus from different messenger RNAs containing defective portions of a virus has been documented by many research groups (Hayes *et al.*, 1989; Choi *et al.*, 2000; Rabindran and Dawson, 2001). Since viral vectors that retain the ability to spread systemically do form infectious viral particles, it is theoretically possible that a transgene carried by a viral replicon might be transmitted to a new plant and incorporated into its genome. Such a transfer has, however, never been detected, and is very unlikely in view of the biology of the vectors currently in use (they are confined to the cytoplasm, and do not have the ability to reverse transcribe their RNA into DNA). Some TMV-based viral vectors engineered to express pharmaceutical proteins have now been used in tobacco fields for several years (by Large Scale Biology Corp, Vacaville, CA, USA). Extensive safety tests have been performed and, as a result, US regulatory agencies are comfortable with controlled open release of such viral vectors. Of course, it is possible to make viral vectors even safer, and this has been a subject of research in our lab for the past few years. In particular, the viral vectors that we are developing do not require systemic movement, and therefore do not encode a coat protein. Therefore, the amplicons are not packaged in a viral particle and cannot be mechanically transmitted to uninfected plants. Rather, we encode in the plant genome an inactive 'encrypted' version of a viral genome that can be activated in various ways. In addition, for a number of technical reasons, codon usage of part of the viral genome was modified. Therefore, this feature should reduce the likelihood of recombination with wild-type viruses.

Epidemiology and mistake correction

Any technical process can potentially get out of control. For transgenic organisms, such an event would have dramatic consequences, since a replicating living organism cannot be recalled. Therefore, in case of uncontrolled release or escape of a GM organism into the environment, it would be useful to incorporate some control elements at the transgenic locus to allow damage control *a posteriori*. In many cases, the simple use of a broad-spectrum herbicide would allow the destruction of undesired volunteers. In other instances, one might want to be able to use a more targeted approach to destroy only the individuals that carry a specific gene. One approach may be to engineer a counter-selectable linked to the gene of interest, such as the bacterial cytochrome P450SU1 (which converts a sulfonylurea pro-herbicide to a cytotoxic metabolite; O'Keefe *et al.*, 1994; Koprek *et al.*, 1999). Schernthaner and colleagues (2003) propose a trait control system that relies on seed-lethal genes (iaaM and iaaH) that cause auxin overproduction. Alternatively, as a passive approach, the GM plant could be engineered to be less fit for survival in an open environment; however, in such case, *inter alia*, one has to find a solution that does not impose a penalty on the productivity of the system.

GM organism/transgene labelling and identity tags

There are many reasons for developing a universal and reliable system for traceability of transgenic organisms and derivative products. One of the reasons is the need of different regulatory and environmental agencies to reliably monitor transgenic organisms and recombinant products. Such a system would, therefore, give consumers the insurance that non-approved GM-derived products are not present in food products. Proposed and existing detection methods are based on the highly sensitive PCR technology, which allows detection of recombinant DNA even in processed derivatives of transgenic organisms (Vollenhofer *et al.*, 2001). However, there is, at present, no generally accepted universal system to ensure easy traceability of transgenic organisms. A universal labelling system that would consist of short DNA tags incorporated to all transgenic organisms was proposed both by us and others (Gressel and Ehrlich, 2002; Marillonnet *et al.*, 2003). According to the system that we proposed, these tags consist of a short DNA sequence that encodes a message using a redundant 'non-genetic code', which could be retrieved easily and read. The artificial code would allow conversion of nucleotide triplets into alphanumerical characters, thus allowing messages to be written in any existing language. The DNA labels would be designed to be biologically neutral, stable through several generations, would be tightly linked with the transgene(s), and would be resistant against corruption (mutation) of encoded technical information.

Other 'good transgene management practices'

Rational design of the construct to be introduced into the plant should exclude any unnecessary DNA sequences that do not play an essential role. This includes vector backbone sequences, as well as regulatory/coding sequences, as these might cause heterologous DNA/expression pattern instability in the host plant. Elements that are incorporated into the vectors or that might serve as helpers at various stages of

engineering, should be removed whenever possible. These might be transformation marker genes, or other 'helper' genes like site-specific recombinases, etc. There are numerous technologies that have been used effectively to eliminate the selection markers from primary transformants, including those that rely on the use of transposition or site-specific recombination, and those that are based on independent integration and segregation of the genes encoding the trait of interest and the selectable marker (to mention but a few: Sugita *et al.*, 2000; Corneille *et al.*, 2001; Zuo *et al.*, 2001; Hoa *et al.*, 2002).

Other management practices dictated by common sense include: 1) tools, elements of 'operating system' should be artificially evolved not to communicate with the living world; 2) genes of interest should be primarily of plant origin; and 3) engineering, production and distribution steps, including early research and development, have to be assessed for inherent versus acceptable risks.

Integrated processes

'The guy who invented the wheel was an idiot. But the guy who invented the other three, he was a genius.' (Sid Ceasar)

Using some of the elements described above, we now attempt to assemble a working scenario for a process that would be both safe and efficient. Here, we choose to address a difficult case, i.e. the production of a highly active molecule in plants. Such activity would require a maximum level of biological isolation (plants of classes III and IV as defined in 'Introduction'). We further assume that cultivation takes place in an open field, in areas with intensive agriculture.

Our choice for such a high security process would be a combination of a hybrid seed system, combining operationally linked trait and germplasm locks. Thus, we limit ourselves to cross-pollinating plants only, including many major crops (corn, rice, canola, sugar beets) as well as some secondary crops (millet, sorghum, chickpea). Any hybrid seed production system involves several steps, all of which have to be controlled. Most systems rely on the use of three parental components: 1) a male sterile line that has to be propagated with the help of 2) a so-called maintainer line; in the final step of hybrid production, the male sterile line is pollinated with a 3) pollinator line. Usually, the hybrid plants grown by a farmer express a trait of interest (hybrid vigour), whereas parental lines do not. For our purpose, instead of unlocking hybrid vigour, hybridization serves two purposes: 1) it serves as a switch to 'unencrypt' an encoded message, leading to production of the molecule of interest; and 2) it leads to activation of processes that render the hybrid seed biologically sterile, i.e. incapable of normal development or incapable of subsequent sexual reproduction. The parental lines have to be genetically modified to incorporate the necessary locks. Designs of such double locks may rely on different mechanisms, but for safety and engineering reasons, they have to be operationally tightly linked to each other (initiation of production and biological sterilization). Furthermore, since the pollinator line will be allowed to shed pollen in an open field, in an area significantly larger than with the maintainer line, we suggest that the genes or gene fragments that code for the enzyme or product of interest are engineered into the male sterile and maintainer lines, whereas the pollinator line would contain only the necessary switches.

This dual control switch may be used for the design of multiple independent production systems. Therefore, the same design may be used for independent processes in nearby fields. For such cases, it is essential that the switch unlocking the production is made to be highly specific, and that its technical design would allow multiple specific unencryption combinations. One possible example of such a switch would be an intein-based *trans*-splicing that allows very specific reassembly and could accommodate numerous unencryption combinations, both through the choice of intein and of the different splicing locations, as well as through engineering of the specificity of the assembled protein (*Figure 14.11*). The 'sterilization' switch, on the other hand, can be much less specific, or almost universal, as its purpose is to genetically neutralize the effect of any intended or illicit 'production cross'. Finally, considerations have to be given to a potential toxicity effect of the proteins expressed in the system prior to unencryption. These may include intein fusions and other expressed proteins or polypeptide fragments in the parental lines.

Last, but not least, the parental lines and hybrids all have to be sensitive to at least one broad-spectrum herbicide, to allow for *a posteriori* damage control of any unintended escape; they also have to be properly labelled at the DNA level and, if practical, also at the level of resulting phenotype (for example, colour of the seeds containing the active product).

Case scenario 1: production of pharmaceutical proteins in tobacco leaves

Under our scenario (which reflects actual technology in development at Icon Genetics, Munich, Germany), we manufacture proteins in tobacco leaves using switchable viral amplicons (*Figure 14.8*). Tobacco is chosen because it is not a food plant, it has no wild relatives in nearby areas, and there are no cultivated tobacco fields within a 10 km radius. Our plants are grown in a greenhouse until maturity, and then the amplification process is activated. The material is then harvested and processed. Special precautions are taken to prevent escape of pollen and seeds from the growth areas: outgoing air is filtered; the growth areas are treated, chemically and physically, to inactivate any undesired live material; biological waste that contains living material is autoclaved. Since amplification of viral vectors takes only 7–14 days, the producing plants and other grown plants are kept separately.

The following biological barriers are considered as appropriate:

(1) male sterility of the host. Using cell fusion between tobacco and wild species *Hyoscyamus*, we produced cytoplasmic hybrids that have tobacco nucleus, whereas other organelles (plastids and mitochondria) are derived from *Hyoscyamus*. Alien mitochondria cause full male sterility of the plants;
(2) lowered fitness of the host if escaped in the open. Our material has high sensitivity to strong light because in the genotype used, plastids are also derived from *Hyoscyamus*;
(3) controlled hybrid seed production. For multiplication of the host, it has to be pollinated with a maintainer line. The maintainer line (which produces pollen) is grown, and the crosses are being performed under tightly controlled conditions. Additionally, the pollinator is engineered so as to not contain any gene directly involved in amplicon formation and amplification;

(4) a defective amplicon design is being adopted: the amplicon is unable to form a mature virus particle. It has been repeatedly shown in field trials that TMV-based RNA viral amplicons, similar to the one we use, rapidly loose any introduced genetic material, and do not survive simple physical treatments. To further reduce this already remote possibility of escape, the amplicons in our material do not contain the gene for coat protein (necessary for systemic movement and efficient mechanical infectivity). The amplicon is generated inside a plant cell, in multiple cells of the plant simultaneously. The DNA encoding our amplicon also contains numerous artificial introns; therefore, any transfer into a bacterium would not result in any expression;

(5) use of encrypted amplicons that cannot be expressed in bacterial systems. The DNA encoding our amplicon contains numerous artificial introns; therefore, any transfer into a bacterium would not result in any expression;

(6) low recombinogenic ability of the amplicon. One detectable event in viral systems is recombination between RNA molecules in a plant cell, especially if the recombination result is amplifiable. Since wild-type viruses cannot be entirely excluded, the RNA of our amplicon has been modified to lower the homology with wild-type viral RNA that may be present in the plant cell;

(7) switchable process. Even in the production host, the amplification process is not starting until a transcriptional activator is provided. The amplification is thus limited to a less than two-week-long period, during which time plants are maintained in a specialized greenhouse with higher biosafety parameters;

(8) identity tags. All transgenic loci carry an identity tag in the form of a DNA sequence carrying technical information.

Case scenario 2: production of high-value proteins/oils in seed of canola

Under this scenario (which, again, reflects real technology under development at Icon Genetics, Munich, Germany), we intend to manufacture protein or specialty oil in canola in open field conditions. The transgenic material for production may be grown in areas where other canola varieties are also grown. The scenario is based on production in hybrid seed, thus an effective hybrid seed system has to be part of the process. Maintenance of the parental lines has to be both practical and safe. The proposed process is generally applicable to any cross-pollinating species.

The following biological barriers are being considered as appropriate:

(1) the process allows for expression of the trait of interest in hybrid seed only. The product is not expressed in parental lines. Also, parental lines do not express any gene that would impede plant growth or plant development, or that would present a biosafety concern during multiplication of parental material;

(2) the male sterile parent (‘mother plant’) in the hybrid system carries a complete version of the gene(s) required for making the product (genes that require tight ‘escape lock’); therefore, no pollen is shed from this parent. The pollinator plant contains a minimum of exogenous genetic material (in our case – an inactive integrase fragment fused to an intein and an inactive barnase fragment, both of which have no functionality on their own). If necessary, all DNA inserts can be made from elements of plant origin;

(3) the process lock that is activated during hybrid seed formation is based on integrase-mediated DNA recombination, which follows integrase formation (by intein-mediated *trans*-splicing of the two fragments present in the parents). Interaction between the two integrase–intein fragments is highly specific, and multiple pairs can be engineered to coexist in the same plant or the same field, without cross-interfering;
(4) the two components of the lock are located at iso-allelic positions on homologous chromosomes; thus, they 'are linked in repulsion', i.e. upon any meiosis, they will segregate to different gametes. This configuration will significantly reduce the probability of a progeny acquiring both components in a stable manner;
(5) upon hybrid seed formation, another pair of genetic components is activated, rendering the seed incapable of normal plant development/sexual reproduction. In our specific case, this lock is based on intein-mediated *trans*-splicing of barnase fragments in developing embryos. Since these gene fragments are placed at the same locus as the first pair ('process switch'), they will always be activated in the seeds that are also activated for production. As a result, a 'production' seed will automatically be unable to become a source of genetic contamination;
(6) all the components can only work together, and require a eukaryotic cell to be able to function. Thus, the risk of transfer of whole system to bacteria becomes statistically negligible;
(7) all parental material, as well as all progeny used in the process, is sensitive to at least one broad-spectrum, non-selective herbicide. This sensitivity allows for killing any 'volunteer', if necessary;
(8) identity tags. All loci are tagged using DNA-based technical information labels.

Acknowledgements

We thank Dr. Leonid Shlumukov for critical reading of this manuscript. We also thank Dr. Stefan Werner for providing pictures for *Figures 14.7* and *14.10*, and Dr. Mario Gils for providing a picture for *Figure 14.8*.

References

AN, Y.Q., MCDOWELL, J.M., HUANG, S., MCKINNEY, E.C., CHAMBLISS, S. AND MEAGHER, R.B. (1996). Strong constitutive expression of Arabidopsis AcT2/ACT8 actin subclass in vegetative tissues. *Plant Journal* **10**, 107–121.

AOYAMA, T. AND CHUA, N.H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant Journal* **11**, 605–612.

AYRE, B.G., KOEHLER, U., GOODMAN, H.M. AND HASELOFF, J. (1999). Design of highly specific cytotoxins by using trans-splicing ribozymes. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3507–3512.

AYRE, B.G., KOEHLER, U., TURGEON, R. AND HASELOFF, J. (2002). Optimization of trans-splicing ribozyme efficiency and specificity by *in vivo* genetic selection. *Nucleic Acids Research* **30**, E141.

BANROQUES, J., DELAHODDE, A. AND JACQ, C. (1986). A mitochondrial RNA maturase gene transferred to the yeast nucleus can control mitochondrial mRNA splicing. *Cell* **46**, 837–844.

BANROQUES, J., PEREA, J. AND JACQ, C. (1987). Efficient splicing of two yeast mitochondrial introns controlled by a nuclear-encoded maturase. *EMBO Journal* **6**, 1085–1091.

BAULCOMBE, D.C. (1999). Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology* **2**, 109–113.
BETZNER, A.S., OAKES, M.P. AND HUTTNER, E. (1997). Transfer RNA-mediated suppression of amber stop codons in transgenic *Arabidopsis thaliana*. *Plant Journal* **11**, 587–595.
BOHNER, S., LENK, I.I., RIEPING, M., HEROLD, M. AND GATZ, C. (1999). Technical advance: transcriptional activator TGV mediates dexamethasone-inducible and tetracycline-inactivatable gene expression. *Plant Journal* **19**, 87–95.
BOTTERMAN, J., MICHIELS, F. AND CORNELISSEN, M. (1996). Method to obtain male-sterile plants. PCT Patent WO9626283.
BOURDON, V., HARVEY, A. AND LONSDALE, D.M. (2001). Introns and their positions affect the translational activity of mRNA in plant cells. *EMBO Reports* **2**, 394–398.
BRIGHT, S.W.J., GREENLAND, A.J., JEPSON. I. AND PAINE, J.A.M. (1994). Improved plant germplasm. PCT Patent WO9403619.
BUCKLAND, A.G. AND WILTON, D.C. (2000). Anionic phospholipids, interfacial binding and the regulation of cell functions. *Biochimica et Biophysica Acta* **1483**, 199–216.
BURGESS, D.G., RALSTON, E.J., HANSON, W.G. *ET AL.* (2002). A novel, two-component system for cell lethality and its use in engineering nuclear male sterility in plants. *Plant Journal* **31**, 113–122.
CADDICK, M.X., GREENLAND, A.J., JEPSON, I. *ET AL.* (1997). An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nature Biotechnology* **16**, 177–180.
CARNEIRO, V.T., PELLETIER, G. AND SMALL, I. (1993). Transfer RNA-mediated suppression of ctop codons in protoplasts and transgenic plants. *Plant Molecular Biology* **22**, 681–690.
CASPER-LINDLEY, C., DAHLBECK, D., CLARK, E.T. AND STASKAWITCZ, B.J. (2002). Direct biochemical evidence for type III secretion-dependent translocation of the AvrBs2 effector protein into plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 8336–8341.
CECH, T. (1995). Group I introns: new molecular mechanisms for mRNA repair. *Biotechnology* **13**, 323–326.
CHAUDHURY, A.M., LUO, M., MILLER, C., CRAIG, S., DENNIS, E.S. AND PEACOCK, W.J. (1997). Fertilisation-independent seed development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 4223–4228.
CHIN, H.G., KIM, G.-D., MARIN, I. *ET AL.* (2003). Protein trans-splicing in transgenic plant chloroplast: reconstitution of herbicide resistance from split genes. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 4510–4515.
CHOI, I.R., STENGER, D.C., MORRIS, T.J. AND FRENCH, R. (2000). A plant virus vector for systemic expression of foreign genes in cereals. *Plant Journal* **23**, 547–555.
CHOISNE, N., MARTIN-CANDELL, A. AND SMALL, I. (1997). Transactivation of a target gene using a suppressor tRNA in transgenic tobacco plants. *Plant Journal* **11**, 597–604.
CHRISTENSEN, A.H. AND QUAIL, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* **5**, 213–218.
COLLMER, A., BADEL, J.L., CHARKOWSKI, A.O. *ET AL.* (2000). *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8770–8777.
CONNER, A.M. AND JACOBS, J.M. (1999). Genetic engineering of crops as a potential source of genetic hazard in the human diet. *Mutation Research* **443**, 223–234.
CORNEILLE, S., LUTZ, K., SVAB, Z. AND MALIGA, P. (2001). Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system. *Plant Journal* **27**, 171–178.
DALE, P.J., CLARKE, B. AND FONTES, E.M.G. (2002). Potential for the environmental impact of transgenic crops. *Nature Biotechnology* **20**, 567–574.
DAMM, B., SCHMIDT, R. AND WILLMITZER, L. (1989). Efficient transformation of *Arabidopsis thaliana* using direct gene transfer to protoplasts. *Molecular and General Genetics* **217**, 6–12.
DANIELL, H. (2002). Molecular strategies for gene containment in transgenic crops. *Nature Biotechnology* **20**, 581–586.

DANIELL, H. AND PARKINSON, C.L. (2003). Jumping genes and containment. *Nature Biotechnology* **21**, 374–375.

DAY, C.D., GALGOCI, B.F. AND IRISH, V.F. (1995). Genetic ablation of petal and stamen primordial to elucidate cell interactions during floral development. *Development* **121**, 2887–2895.

DENIS, M., DELOURME, R., GOURETT, J.P., MARIANI, C. AND RENARD, M. (1993). Expression of engineered nuclear male sterility in *Brassica napus* (genetics, morphology, cytology, and sensitivity to temperature). *Plant Physiology* **101**, 1295–1304.

DE VRIES, J., HEINE, M., HARMS, K. AND WACKERNAGEL, W. (2003). Spread of recombinant DNA by roots and pollen of transgenic potato plants, identified by highly specific biomonitoring using natural transformation of an Acinetobacter sp. *Applied and Environmental Microbiology* **69**, 4455–4462.

DONSON, J., KEARNEY, C.M., HILF, M.E. AND DAWSON, W.O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 7204–7208.

DU JARDIN, P., PORTETELLE, D., HARVENGT, L., DUMONT, M. AND WATHELET, B. (1994). Expression of intron-encoded maturase-like polypeptides in potato chloroplasts. *Current Genetics* **25**, 158–163.

EBINUMA, H., SUGITA, K., MATSUNAGA, E. AND YAMAKADO, M. (1997). Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 2117–2121.

ELLSTRAND, N.C. (2003). Going to 'great lengths' to prevent the escape of genes that produce specialty chemicals. *Plant Physiology* **132**, 1770–1774.

ENDO, S., SUGITA, K., SAKAI, M., TANAKA, H. AND EBINUMA, H. (2002). Single-step transformation for generating marker-free transgenic rice using the ipt-type MAT vector system. *Plant Journal* **30**, 115–122.

FABIJANSKI, S.F., ARNISON, P.G., ROBERT, L.S. AND SCHERNTHANER, J.P. (1999). PCT Patent WO 00/37660.

FALK, M.C., CHASSY, B.M., HARLANDER, S.K., HOBAN, T.J., MCGLOUGHLIN, M.N. AND AKHLAGHI, A.R. (2002). Food biotechnology: benefits and concerns. *Journal of Nutrition* **132**, 1384–1390.

FITZMAURICE, W.P., HOLZBERG, S., LINDBO, J.A. *ET AL.* (2002). Epigenetic modification of plants with systemic RNA viruses. *OMICS A Journal of Integrative Biology* **6**, 137–151.

FRANCK, A., GUILLEY, H., JONARD, G., RICHARDS, K. AND HIRTH, L. (1980). Nucleotide sequence of cauliflower mosaic virus DNA. *Cell* **21**, 285–294.

FUTAKI, S., SUZUKI, T., OHASHI, W. *ET AL.* (2001). Arginine-rich peptides. *Journal of Biological Chemistry* **276**, 5836–5840.

GARCIA-BLANCO, M.A. (2003). Messenger RNA reprogramming by spliceosome-mediated RNA trans-splicing. *Journal of Clinical Investigation* **112**, 474–480.

GATZ, C. (1997). Chemical control of gene expression. *Annual Review of Plant Physiology and Molecular Biology* **48**, 89–108.

GATZ, C. AND QUAIL, P.H. (1988). *Tn 10*-encoded Tet repressor can regulate an operator-containing plant promoter. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 1394–1397.

GATZ, C., FROHBERG, C. AND WENDENBURG, R. (1992). Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant Journal* **2**, 397–404.

GOGARTEN, J.P., SENEJANI, A.G., ZHAXYBAYEVA, O., OLENDZENSKI, L. AND HILARIO, E. (2002). Inteins: structure, function and evolution. *Annual Review of Microbiology* **56**, 263–287.

GOLDMAN, M.H., GOLDBERG, R.B. AND MARIANI, C. (1994). Female-sterile tobacco plants are produced by stigma-specific cell ablation. *EMBO Journal* **13**, 2976–2984.

GRATTON, J.P., YU, J., GRIFFITH, J.W. *ET AL.* (2003). Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and *in vivo*. *Nature Medicine* **9**, 357–362.

GRAY, J., PICTON, S., SHABBEER, J., SCHUCH, W. AND GRIERSON, D. (1992). Molecular biology of fruit ripening and its manipulation with antisense genes. *Plant Molecular Biology* **19**, 69–87.

GRESSEL, J. AND EHRLICH, G. (2002). Universal inheritable barcodes for identifying organisms. *Trends in Plant Science* **7**, 542–544.

GRITZ, L. AND DAVIES, J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**, 179–188.

HAM, J.H., BAUER, D.W., FOUTS, D.E. AND COLLMER, A. (1998). A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 10206–10211.

HAN, K., JEON, M.J., KIM, K.A., PARK, J. AND CHOI, S.Y. (2000). Efficient intracellular delivery of GFP by homeodomains of *Drosophila Fushitarazu* and Engrailed proteins. *Molecules and Cells* **10**, 728–732.

HANSON, B., ENGLER, D., MOY, Y., NEWMAN, B., RALSTON, E. AND GUTTERSON, N. (1999). A simple method to enrich an *Agrobacterium*-transformed population for plants containing only T-DNA sequences. *Plant Journal* **19**, 727–734.

HAYES, R.J., COUTTS, R.H. AND BUCK, K.W. (1989). Stability and expression of bacterial genes in replicating geminivirus vectors in plants. *Nucleic Acids Research* **17**, 2391–2403.

HOA, T.T., BONG, B.B., HUQ, E. AND HODGES, T.K. (2002). Cre/lox site-specific recombination controls the excision of a transgene from the rice genome. *Theoretical and Applied Genetics* **104**, 518–525.

HUANG, C.Y., AYLIFFE, M.A. AND TIMMIS, J.N. (2003). Direct measurement of the transfer rate of chloroplast DNA into the nucleus. *Nature* **422,** 72–76.

JAEGER, G.D., SCHEFFER, S., JACOBS, A. *ET AL.* (2002). Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nature Biotechnology* **20**, 1265–1268.

JOHANSEN, I.E. (1996). Intron insertion facilitates amplification of cloned virus cDNA in *Escherichia coli* while biological activity is re-established after transcription *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 12400–12405.

JUNKER, B.H., CHU, C., SONNEWALD, U., WILLMITZER, L. AND FERNIE, A.R. (2003). In plants the alc gene expression system responds more rapidly following induction with acetaldehyde than with ethanol. *FEBS Letters* **535**, 136–140.

KAPILA, J., DE RYCKE, R., VAN MONTAGU, M. AND ANGENON, G. (1997). An *Agrobacterium*-mediated gene expression system for intact leaves. *Nucleic Acids Research* **31**, 826–832.

KAPUSCINSKI, A.R., GOODMAN, R.M., HANN, S.D. *ET AL.* (2003). Making 'safety first' a reality for biotechnology products. *Nature Biotechnology* **21**, 599–601.

KARIMOVA, G., PIDOUX, J., ULLMANNN, A. AND LADANT, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5752–5756.

KAY, E., VOGEL, T.M., BERTOLLA, F., NALIN, R. AND SIMONET, P. (2002). *In situ* transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Applied and Environmental Microbiology* **68**, 3345–3351.

KLIMYUK, V.I., IVANOV, P., MARILLONNET, S., ZENK, M. AND GLEBA, Y. (2002). Amplification vectors based on trans-splicing. PCT Patent WO 02097080.

KOLTUNOW, A.M. (1993). Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. *Plant Cell* **5**, 1425–1437.

KOLTUNOW, A.M., BICKNELL, R.A. AND CHAUDHURY, A.M. (1995). Molecular strategies for the generation of genetically identical seeds without fertilization. *Plant Physiology* **108**, 1345–1352.

KONDO, K., YAMAMOTO, M., ITAHASHI, R. *ET AL.* (2002). Insight into the evolution of self-compatibility in Lycopersicon from a study of stylar factors. *The Plant Journal* **30**, 143–153.

KOPREK, T., MCELROY, D., LOUWERSE, J., WILLIAMS-CARRIER, R. AND LEMAUX, P.G. (1999). Negative selection system for transgenic barley (*Hordeum vulgare* L.): comparison of bacterial coda- and cytochrome P450 gene-mediated selection. *Plant Journal* **19**, 719–726.

KUMAGAI, M.H., TURPEN, T.H., WEINZETTL, N. *ET AL.* (1993). Rapid, high-level expression of

biologically active alpha-trichosanthin in transfected plants by an RNA viral vector. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 427–430.

KUMAGAI, M.H., DONSON, J., DELLA-CIOPPA, G., HARVEY, D., HANLEY, K. AND GRILL, L.K. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 1679–1683.

KUMAGAI, M.H., DONSON, J., DELLA-CIOPPA, G. AND GRILL, L. (2000). Rapid, high-level expression of glycosylated rice alpha-amylase in transfected plants by an RNA viral vector. *Gene* **245**, 169–174.

KUNKEL, T., NIU, Q.W., CHAN, Y.S. AND CHUA, N.H. (1999). Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. *Nature Biotechnology* **17**, 916–919.

KUVSHINOV, V.V., KOIVU, K., KANERVA, A. AND PEHU, E. (2001). Molecular control of transgene escape from genetically modified plants. *Plant Science* **160**, 517–522.

KWON, D., CHALMERS, R.M. AND KLECKER, N. (1995). Structural domains of IS10 transposase and reconstitution of transposition activity from proteolytic fragments lacking an interdomain linker. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8234–8238.

LACK, G. (2002). Clinical risk assessment of GM foods. *Toxicology Letters* **127**, 337–340.

LAN, N., HOWREY, R.P., LEE, S.W., SMITH, C.A. AND SULLENGER, B.A. (1998). Ribozyme-mediated repair of sickle beta-globin mRNAs in erythrocyte precursors. *Science* **280**, 1593–1596.

LIU, X.Q. AND YANG, J. (2003). Split dnaE genes encoding multiple novel inteins in *Trichodesmium erythraeum*. *Journal of Biological Chemistry* **278**, 26315–26318.

LOPEZ-MOYA, J.J. AND GARCIA, J.A. (2000). Construction of stable and highly invectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Research* **68**, 99–107.

LUO, H. AND KAUSCH, A.P. (2002). Application of FLP/FRT site-specific DNA recombination system in plants. *Genetic Engineering (N.Y.)* **24**, 1–16.

LUO, M., BILODEAU, P., KOLTUNOW, A., DENNIS, E., PEACOCK, W.J. AND CHAUDHURY, A.M. (1999). Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 296–301.

LYZNIK, L.A., RAO, K.V. AND HODGES, T.K. (1996). FLP-mediated recombination of FRT sites in the maize genome. *Nucleic Acids Research* **24**, 3784–3789.

MALIGA, P. (2003a). Progress towards commercialization of plastid transformation technology. *Trends in Biotechnology* **21**, 20–28.

MALIGA, P. (2003b). Plant biology: mobile plastid genes. *Nature* **422**, 31–32.

MALLORY, A.C., PARKS, G., ENDRES, M.W. *ET AL.* (2002). The amplicon-plus system for high-level expression in transgenic plants. *Nature Biotechnology* **20**, 622–625.

MARILLONNET, S., KLIMYUK, V. AND GLEBA, Y. (2003). Encoding technical information in GM organisms. *Nature Biotechnology* **21**, 224–226.

MARILLONNET, S., GIRITCH, A., GILS, M., KANDZIA, R., KLIMYUK, V. AND GLEBA, Y. (2004). *In planta* engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6852–6857.

MASCARENHAS, D., METTLER, I.J., PIERCE, D.A. AND LOWE, H.W. (1990). Intron-mediated enhancement of heterologous gene expression in maize. *Plant Molecular Biology* **15**, 913–920.

MATSUSHITA, M., TOMIZAVA, K., MORIWAKI, A., LI, S.T., TERADA, H. AND MATSUI, H. (2001). A high-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation. *Journal of Neuroscience* **21**, 6000–6007.

MAZODIER, P., COSSART, P., GIRAUD, E. AND GASSER, F. (1985). Completion of the nucleotide sequence of the central region of Tn5 confirms the presence of three resistance genes. *Nucleic Acids Research* **13**, 195–205.

MCELROY, D., ZHANG, W., CAO, J. AND WU, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* **2**, 163–171.

MCNELLIS, T.W., MUDGETT, M.B., LI, K. *ET AL.* (1998). Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death. *Plant Journal* **14**, 247–257.

MEIJER, E.G., SCHILPEROORT, R.A., RUEB, S., VAN OS-RUYGROK, P.E. AND HENSGENS, L.A. (1991). Transgenic rice cell lines and plants: expression of transferred chimeric genes. *Plant Molecular Biology* **16**, 807–820.

MERKLE, S.A AND DEAN, J.F. (2000). Forest tree biotechnology. *Current Opinion in Biotechnology* **11**, 298–302.

METT, V.L., LOCHHEAD, L.P. AND REYNOLDS, P.H. (1993). Copper-controllable gene expression system for whole plants. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 4567–4571.

MI, Z., MAI, J., LU, X. AND ROBBINS, P.D. (2000). Characterization of a class of cationic peptides able to facilitate efficient protein transduction *in vitro* and *in vivo*. *Molecular Therapy* **2**, 339–347.

MIKHEEVA, S. AND JARRELL, K.A. (1996). Use of engineered ribozymes to catalyse chimeric gene assembly. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7486–7490.

MLYNAROVA, L. AND NAP, J.P. (2003). A self-excising Cre recombinase allows efficient recombination of multiple ectopic heterospecific lox sites in transgenic tobacco. *Transgenic Research* **12**, 45–57.

MOHR, G. AND LAMBOWITZ, A.M. (2003). Putative proteins related to group II intron reverse transcriptase/maturases are encoded by nuclear genes in higher plants. *Nucleic Acids Research* **31**, 647–652.

MOR, T.S., MOON, Y.S., PALMER, K.E. AND MASON, H.S. (2003). Geminivirus vectors for high-level expression of foreign proteins in plant cells. *Biotechnology and Bioengineering* **81**, 430–437.

MURGOLA, E.J. (1985). tRNA, suppression and the code. *Annual Review of Genetics* **19**, 57–80.

NAP, J.-P., METZ, P.L., ESCALER, M. AND CONNER, A.J. (2003). The release of genetically modified crops into the environment. Part I. Overview of current status and regulations. *Plant Journal* **33**, 1–18.

O'KEEFE, D.P., TEPPERMAN, J.M., DEAN, C., LETO, K.J., ERBES, D.L. AND ODELL, J.T. (1994). Plant expression of a bacterial cytochrome P450 that catalyses activation of sulfonylurea proherbicide. *Plant Physiology* **105**, 473–482.

OLIVER, M.J., QUISENBERRY, J.E., TROLINDER, N.L.G. AND KEIM, D.L. (1999). Control of plant gene expression. U.S. Patent 5,977,441.

ONOUCHI, H., NISHIHAMA, R., KUDO, M., MACHIDA, Y. AND MACHIDA, C. (1995). Visualization of site-specific recombination catalysed by a recombinase from *Zygosaccharomyces rouxii* in *Arabidopsis thaliana*. *Molecular and General Genetics* **247**, 653–660.

OW, D.W. (2002). Recombinase-directed plant transformation for the post-genomic era. *Plant Molecular Biology* **48**, 183–200.

PADIDAM, M. (2003). Chemically regulated gene expression in plants. *Current Opinion in Plant Biology* **6**, 169–177.

PADIDAM, M., GORE, M., LU, D.L. AND SMIRNOVA, O. (2003). Chemical-inducible, ecdysone receptor-based gene expression system for plants. *Transgenic Research* **12**, 101–109.

PEITZ, M., PFANNKUCHE, K., RAJEWSKY, K. AND EDENHOFER, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 4489–4494.

PELLETIER, J.N., CAMPBELL-VALOIS, F.-X. AND MICHNICK, S.W. (1998). Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 12141–12146.

PELLETIER, J.N., ARNDT, K.M., PLUECKTHUN, A. AND MICHNICK, S.W. (1999). An *in vivo* library-versus-library selection of optimized protein–protein interactions. *Nature Biotechnology* **17**, 683–690.

PERERA, R.J., LINARD, C.G. AND SIGNER, E.R. (1993). Cytosine deaminase as a negative selective marker for *Arabidopsis*. *Plant Molecular Biology* **24**, 793–799.

PERLAK, F.J., STONE, T.B., MUSKOPF, Y.M. *ET AL.* (1993). Genetically improved potatoes: protection from damage by Colorado potato beetles. *Plant Molecular Biology* **22**, 313–321.

PERLER, F.B. (1998). Protein splicing of inteins and hedgehog autoproteolysis: structure, function, and evolution. *Cell* **92**, 1–4.

PERLER, F.B. AND ADAM, E. (2000). Protein splicing and its applications. *Current Opinion in Biotechnology* **11**, 377–383.

PERRIMAN, R., BRUENING, G., DENNIS, E.S. AND PEACOCK, W.J. (1995). Effective ribozyme delivery in plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6175–6179.

PORTA, C. AND LOMONOSSOFF, G. (1996). Use of viral replicons for the expression of genes in plants. *Molecular Biotechnology* **5**, 209–221.

PUTTARAJU, M., JAMISON, S.F., MANSFIELD, S.G., GARCIA-BLANCO, M.A. AND MITCHELL, L.G. (1999). Spliceosome-mediated RNA trans-splicing as a tool for gene therapy. *Nature Biotechnology* **17**, 246–252.

RABINDRAN, S. AND DAWSON, W.O. (2001). Assessment of recombinants that arise from the use of a TMV-based transient expression vector. *Virology* **284**, 182–189.

REAM, W. (1998). Import of *Agrobacterium tumefaciens* virulence proteins and transferred DNA into plant cell nuclei. *Subcellular Biochemistry* **29**, 365–384.

RIEGER, M.A., LAMOND, M., PRESTON, C., POWLES, S.B. AND ROUSH, R.T. (2002). Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science* **296**, 2386–2389.

ROSE, A.B. (2002). Requirements for an intron-mediated enhancement of gene expression in *Arabidopsis*. *RNA* **8**, 1444–1453.

ROSLAN, H.A., SALTER, M.G., WOOD, C.D. *ET AL.* (2001). Characterization of the ethanol-inducible alc gene-expression system in *Arabidopsis thaliana*. *Plant Journal* **28**, 225–235.

ROTHSTEIN, S.J., LAHNERS, K.N., LOTSTEIN, R.J., COROZZI, N.B., JAINE, S.M. AND RICE, D.A. (1987). Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. *Gene* **53**, 153–161.

SA, G.M., MI, M., HE-CHUN, Y. AND GUO-FENG, L. (2002). Anther-specific expression of ipt gene in transgenic tobacco and its effect on plant development. *Transgenic Research* **11**, 269–278.

SALDANHA, R., MOHR, G., BELFORT, M. AND LAMBOWITZ, A.M. (1993). Group I and group II introns. *FASEB Journal* **7**, 15–24.

SALTER, M.G., PAINE, J.A., RIDDELL, K.V. *ET AL.* (1998). Characterization of the ethanol-inducible alc gene expression system for transgenic plants. *Plant Journal* **16**, 127–132.

SANCHO, J. AND FERSHT, A.R. (1992). Dissection of an enzyme by protein engineering. The N- and C-terminal fragments of barnase form a native-like complex with restored enzymatic activity. *Journal of Molecular Virology* **224**, 741–747.

SANTORO, S.W. AND SCHULTZ, P.G. (2002). Directed evolution of the site-specificity of Cre recombinase. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 4185–4190.

SCHERNTHANER, J.P., FABIJANSKI, S.F., ARNISON, P.G., RACICOT, M. AND ROBERT, L.S. (2003). Control of seed germination in transgenic plants based on the segregation of a two-component system. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 6855–6859.

SCHWARTZ, J.J. AND ZHANG, S. (2000). Peptide-mediated cellular delivery. *Current Opinion in Molecular Therapeutics* **2**, 162–167.

SERRANO, L., DAY, A.G. AND FERSHT, A.R. (1993). Step-wise mutation of barnase to binase. A procedure for engineering increased stability of proteins and an experimental analysis of the evolution of protein stability. *Journal of Molecular Evolution* **233**, 305–312.

SHINGLEDECKER, K., JIANG, S.Q. AND PAULUS, H. (1998). Molecular dissection of the Mycobacterium tuberculosis RecA intein: design of a minimal intein and of a trans-splicing system involving two intein fragments. *Gene* **207**, 187–195.

SRIVASTAVA, V. AND OW, D.W. (2001). Single copy primary transformants of maize obtained

through the co-introduction of a recombinase-expressing construct. *Plant Molecular Biology* **46**, 561–566.

SRIVASTAVA, V., ANDERSON, O.D. AND OW, D.W. (1999). Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 11117–11121.

STANLEY, J. (1993). Geminiviruses: plant viral vectors. *Current Opinion in Genetics and Development* **3**, 91–96.

STEGEMANN, S., HARTMANN, S., RUF, S. AND BOCK, R. (2003). High-frequency gene transfer from the chloroplast genome to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 8612–8614.

SUGITA, K., KASAHARA, T., MATSUNAGA, E. AND EBINUMA, H. (2000). A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. *Plant Journal* **22**, 461–469.

SULLENGER, B.A. AND CECH, T.R. (1993). Tethering ribozymes to a retroviral packaging signal for destruction of viral RNA. *Science* **262**, 1566–1569.

SULLENGER, B.A. AND CECH, T.R. (1994). Ribozyme-mediated repair of defective mRNA by targeted trans-splicing. *Nature* **371**, 619–622.

SZUREK, B., ROSSIER, O., HAUSE, G. AND BONAS, U. (2002). Type III-dependent translocation of the Xanthomonas AvrBs2 protein into the plant cell. *Molecular Microbiology* **46**, 13–23.

THOMPSON, C.J. AND GRAY, G.S. (1983). Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5190–5194.

TSUGEKI, R. AND FEDOROFF, N.V. (1999). Genetic ablation of root cap cells in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 12941–12946.

VANCANNEYT, G., SCHMIDT, R., O'CONNOR-SANCHES, A., WILLMITZER, L. AND ROCHA-SOSA, M. (1990). Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Molecular and General Genetics* **220**, 245–250.

VAN DER ENT, F.M.I., VOS, A. AND PLASTERK, R.H.A. (1999). Dissecting the role of the N-terminal domain of Human Immunodeficiency Virus integrase by *trans*-complementation analysis. *Journal of Virology* **73**, 3176–3186.

VAN DIJK, P.J. (2003). Ecological and evolutionary opportunities of apomixes: insights from *Taraxacum* and *Chondrilla*. *Philosophical Transactions of the Royal Society of London. Series B. Biological Sciences* **358**, 1113–1121.

VAN DIJK, P. AND VAN DAMME, J. (2000). Apomixis technology and the paradox of sex. *Trends in Plant Science* **5**, 81-84.

VAQUERO, C., SACK, M., CHANDLER, J. *ET AL.* (1999). Transient expression of a tumour-specific single chain fragment and a chimeric antibody in tobacco leaves. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 11128–11133.

VERGUNST, A.C., SCHRAMMEIJER, B., DEN DULK-RAS, A., DE VLAAM, C.M.T., REGENSBURG-TUINK, T.J.G. AND HOOYKAAS, P.J.J. (2000). VirB/D4-dependent protein translocation from *Agrobacterium* into plant cell. *Science* **290**, 979–982.

VOGEL, J., HUBSCHMANN, T., BORNER, T. AND HESS, W.R. (1997). Splicing and intron-internal RNA editing of trnK-matK transcripts in barley plastids: support for MatK as an essential splice factor. *Journal of Molecular Biology* **270**, 179–187.

VOINNET, O., RIVAS, S., MESTRE, P. AND BAULCOMBE, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant Journal* **33**, 949–956.

VOLLENHOFER, S., BURG, K., SCHMIDT, J. AND KROATH, H. (2001). Genetically modified organisms in food-screening and specific detection by polymerase chain reaction. *Journal of Agricultural and Food Chemistry* **47**, 5038–5043.

WAHLEITHNER, J.A., MACFARLANE, J.L. AND WOLSTENHOLME, D.R. (1990). A sequence encoding a maturase-related protein in a group II intron of a plant mitochondrial nad1 gene.

Proceedings of the National Academy of Sciences of the United States of America **87**, 548–552.

WALTERS, D.A., VETSCH, C.S., POTTS, D.E. AND LUNDQUIST, R.C. (1992). Transformation and inheritance of hygromycin phosphotransferase gene in maize plants. *Plant Molecular Biology* **18**, 189–200.

WEINMANN, P., GOSSEN, M., HILLEN, W., BUJARD, H. AND GATZ, C. (1994). A chimeric transactivator allows tetracycline-responsive gene expression in whole plants. *Plant Journal* **5**, 559–569.

WELD, R., HEINEMANN, J. AND EADY, C. (2001). Transient GFP expression in *Nicotiana plumbaginifolia* suspension cells: the role of gene silencing, cell death and T-DNA loss. *Plant Molecular Biology* **45**, 377–385.

WENDER, P.A., MITCHELL, D.J., PATTABIRAMAN, K., PELKEY, E.T., STEINMAN, L. AND ROTHBARD, J.B. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13003–13008.

WERNER, S., GIRITCH, A., ELIBY, S., MARILLONNET, S., KLIMYUK, V. AND GLEBA, Y. (2003). Transgenic plants with controlled distribution of a trait to progeny. PCT Patent WO03102197.

WICKELGREN, I. (2003). Spinning junk into gold. *Science* **300**, 1646–1649.

WILLIAMS, M. AND LEEMANS, J. (1998). Maintenance of male-sterile plants. US Patent 5,750,867.

WU, H., HU, Z. AND LIU, X.-Q. (1998a). Protein *trans*-splicing by a split intein encoded in a split DnaE gene of *Synechocystis* sp. PCC6803. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9226–9231.

WU, H., XU, M.Q. AND LIU, X.Q. (1998b). Protein trans-splicing and functional mini-inteins of a cyanobacterial dnaB intein. *Biochimica et Biophysica Acta* **1387**, 422–432.

YANG, J., FOX, G.C. AND HENRY-SMITH, T.V. (2003). Intein-mediated assembly of a functional –glucuronidase in transgenic plants. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 3513–3518.

YANG, S.J., REVERS, F., SOUCHE, S. *ET AL.* (1998). Construction of full-length cDNA clones of lettuce mosaic virus (LMV) and the effect of intron insertion on their viability in *Escherichia coli* and on their infectivity to plants. *Archives of Virology* **143**, 2443–2451.

ZHAO, Y., LOU, D., BURKETT, J. AND KOHLER, H. (2001). Chemical engineering of cell penetrating antibodies. *Journal of Immunological Methods* **254**, 137–145.

ZUBKO, E., SCUTT, C. AND MEYER, P. (2000). Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. *Nature Biotechnology* **18**, 442–445.

ZUO, J., NIU, Q.W., MOLLER, S.G. AND CHUA, N.H. (2001). Chemical-regulated, site-specific DNA excision in transgenic plants. *Nature Biotechnology* **19**, 157–161.