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Research review paper

Inducible gene expression systems and plant biotechnology

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ABSTRACT

Plant biotechnology relies heavily on the genetic manipulation of crops. Almost invariantly, the gene of interest is expressed in a constitutive fashion, although this may not be strictly necessary for several applications. Currently, there are several regulatable expression systems for the temporal, spatial and quantitative control of transgene activity. These molecular switches are based on components derived from different organisms, which range from viruses to higher eukaryotes. Many inducible systems have been designed for fundamental and applied research and since their initial development, they have become increasingly popular in plant molecular biology.

This review covers a broad number of inducible expression systems examining their properties and relevance for plant biotechnology in its various guises, from molecular breeding to pharmaceutical and industrial applications. For each system, we examine some advantages and limitations, also in relation to the strategy on which they rely. Besides being necessary to control useful genes that may negatively affect crop yield and quality, we discuss that inducible systems can be also used to increase public acceptance of GMOs, reducing some of the most common concerns. Finally, we suggest some directions and future developments for their further diffusion in agriculture and biotechnology.

Contents

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1. Introduction

The use of a transgenic approach to study biochemical and biological functions has been of interest in plant science since the first report of plant genetic transformation. In addition, the availability of different protocols for transformation and regeneration of several species has widened the field of the potential applications of transgene technology, which currently range from many areas of basic research to applied biotechnology and crop breeding. Since Genetically Modified (GM) plants reached the markets during the mid-1990s, the world area of biotech crops has increased for thirteen consecutive years at an average growth rate of 12%. In 2008 transgenic crops were grown in 125 million hectares by 13.3 million farmers in 25 countries, in both developed and developing countries (James, 2009). This success is mainly based on two traits, insect resistance and herbicide tolerance. Notably, one common feature of commercially approved GMOs is that the transgene of interest is almost invariably expressed under the control of a constitutive promoter, typically the viral CaMV 35S RNA promoter, or less frequently the nopaline synthase (nos) promoter from *Agrobacterium*. In monocots, classic alternatives are promoters derived from actin and ubiquitin genes. A recent exception is represented by Golden Rice(s), in which transgenes are expressed under the control of the rice endosperm-specific glutelin promoter. Similarly, in the High-Lysine Corn, which is in the pre-launch phase, the cordapA coding sequence is under the control of the maize Globulin 1 promoter, to direct the expression only in the maize germ.

While constitutive expression was proven to be applicable in many instances, not all genes can be ectopically transcribed in plants in a constitutive fashion. Examples include genes encoding for endogenous proteins with a lethal or highly detrimental dominant negative phenotype, and genes that introduce severe modifications of metabolic or developmental pathways that inhibit plant transformation, regeneration or growth. In these cases, the temporal and spatial control of expression is a necessity to gain information about the effects of such genes. Commonly, this class of genes has limited interest for biotech crops. Up to date, commercial GM plants are gain-of-function dominant mutants, in which it is usually acceptable that the transgene is expressed at high level in all plant tissues and phenological phases. Nonetheless, as demonstrated by the Golden Rice and the High-Lysine Corn, controlled expression is taking place in the second generation GMOs. Furthermore, the continuous development of bioreactors for plant cells and green-algae cultures is making more feasible the commercial production of recombinant proteins or algal fuels (Chisti, 2007; Demain and Vaishnav, 2009). These areas represent rapidly developing fields in which chemical regulated promoters can provide obvious advantages.

Inducible gene expression systems for plant molecular biology have been comprehensively reviewed (Moore et al., 2006; Padidam, 2003; Tang et al., 2004; Wang et al., 2003). This article reviews a number of plant systems examining their properties and relevance for plant biotechnology (Fig. 1). We also discuss some advantages and limitations of the strategy on which they rely, and suggest some directions and future developments for their application in plant biotechnology.

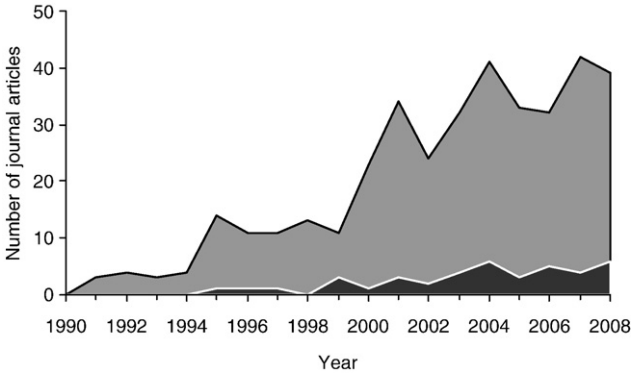

Fig. 1. Trends in literature for inducible gene expression systems in plant science and biotechnology. Number of journal articles (including reviews) per year obtained using fields as search term at the ISI Web of Science, Science Citation Index Expanded (black line above the light grey area). Fields were: "inducible system(s) and plant(s); "inducible expression system(s)" and plant(s); "inducible promoter(s) and plant(s)" "chemical inducible" and plant(s) and expression; "chemical regulated" and plant(s) and expression. The white line above the dark grey area indicates the number of journal articles citing "biotechnology" in their title, abstract, keywords or journal title.

2. Alternatives to constitutive promoters: tissue and cell-type specific promoters, binary systems and recombinase-based approaches to control gene expression

Arguably, the most common alternative to the constitutive expression of a transgene is based on the use of tissue-specific promoters. These regulatory sequences can provide some advantages, but many have an increased background activity during plant regeneration. Furthermore, by definition, the expression of the transgene of interest will be limited to one type of tissue, which may be unsuitable for many applications. Another limitation is that it is not possible to activate and quantitatively control the level of expression. Under this perspective, tissue and cell-type specific promoters are not exogenously inducible and hence, will not be covered in detail in this article (for a review see Potenza et al. (2004)).

Another possibility to control transgene activity is represented by expression systems that are based on two separate elements, which can be introduced in independent lines and subsequently combined by crossing (Betzner et al., 1997; Guyer et al., 1998; Moore et al., 1998). Considering the wealth of knowledge in prokaryotic molecular genetics, the first systems were based on the capacity of a suppressor tRNA to allow translation of a transgenic mRNA containing a premature termination codon. One of these systems, initially established for cell cultures (Franklin et al., 1992), has been shown to function in stably transformed tobacco (Choisne et al., 1997) and *Arabidopsis* (Betzner et al., 1997). This approach was used to conditionally transactivate an amber mutated male sterility gene in *Arabidopsis* but some significant cytotoxic effects were observed during tissue culture and in mature plant (Betzner et al., 1997). Even if the nature of such abnormalities was not clarified, it is likely that the modified suppressor tRNA may alter in an unpredictable way endogenous gene expression. However, it is still puzzling that these effects were not observed in tobacco (Choisne et al., 1997).

The other binary systems that have been developed are based on the combination of a synthetic transcription factor with a promoter (containing the corresponding binding site) that controls the expression of the gene of interest. One system used the DNA binding domain of Gal4 from *S. cerevisiae* fused to the transcription activation domain of maize C1. This transcription factor was constitutively expressed in *Arabidopsis* and showed to specifically transactivate reporter expression when crossed to a second transgenic line containing a synthetic promoter bearing Gal4 binding sites (Guyer et al., 1998). An alternative system is based on a chimeric transcription factor LhG4, which consists of the transcription activation domain II from Gal4 of *S. cerevisiae* fused to a modified version of the *E. coli* lac repressor. The lac repressor sequence used carries a tyrosine to histidine mutation that has been shown to increase the binding affinity of repressor for operator by 100 fold, with little accompanying loss in specificity (Lehming et al., 1987). The cis-controlling sequence for the gene of interest is made up of two modified *lac* operators and a minimal 35S promoter (+8 to −50).

The strategy of combining activator and reporter in a hybrid plant is valuable for particular applications and carries significant advantage

over constitutive expression of genes that strongly affect plant physiology. In its classic form, it offers little or no temporal control, as the transcription factor is controlled by a constitutive promoter. More recently, it has been demonstrated that, by selecting appropriate lines, it also possible to achieve temporal and spatial control of transgene activity by limiting the expression to certain parts of the plant with the use of a tissue specific promoter (Baroux et al., 2005; Brand et al., 2006; Chaturvedi et al., 2007). In these instances, a binary system may also allow the coordinated expression of more than one transgene in a predictable way, exploiting a single activator transgenic event in which the expression pattern of the promoter has been well characterised (Moore et al., 1998). Finally, besides its relevance for functional studies, a further advantage of this type of binary systems is that the common leakiness of tissue specific promoters during plant transformation and regeneration would not represent a problem.

Binary activator systems may be valuable in agriculture, where the production of F_1 hybrid lines is often favoured by breeding companies. Currently, this kind of controlled expression system has not yet found a commercial application. One reason may be that a hybrid cultivated variety cannot be propagated by farmers *per se*, and hence, a two-component inducible system will not represent a novel desirable feature. The same argument can be reversed for self pollinating plants. In this case, the presence of the two elements of the expression system in a commercial line will be easily preserved by selfing, making irrelevant the binary nature of the expression system. An important area of application is represented by the control of genes that strongly affect plant phenotype. Obvious examples of interest for plant biotechnology are genes that inhibit gamete development or seed germination, which may be exploited to obtain commercial (male) sterile plants or seeds that do not germinate, respectively. However, while the first approach may be applicable to a restricted number of feed crops to limit the dispersal of transgenic pollen, the latter will be prone to a number of ethical criticisms (Conway, 2000). Binary systems have also some functional restrictions. As their elements are introduced in two different plants that are subsequently crossed for transactivation, they cannot be applied to plants that are vegetatively propagated. Finally, as mentioned before, there is no need or possibility to externally control the induction in the hybrid plants and quantitative control of the gene activity is not easily achievable.

Similar functional limitations are displayed by inducible gene expression systems that are based on Cre-*lox* (Bayley et al., 1992; Hoff et al., 2001) or alternative recombination systems (Estruch et al., 1991). These site-specific recombination systems consist of two components: i) a recombination enzyme and ii) two short DNA sequences, specifically bound by the recombinase, which flank the DNA region to be excised. These two elements are sufficient to carry out recombination reactions in heterologous systems, thus enabling a variety of applications such as site-specific integration, copy number reduction, and marker gene removal in both the nuclear and plastid genome (Corneille et al., 2001; Gidoni et al., 2008). It is conceivable that recombinase-based systems would be primarily used in plant biotechnology as a valuable tool for controlled marker-excision in transgenic crops (Hare and Chua, 2002). However, a recombinase-based two component system possesses some limitations, as it requires additional breeding efforts to get rid of the recombinase gene and it is not applicable to vegetatively propagated crops. For these reasons, developmentally regulated or heat-inducible gene promoters have been used to control recombinase expression, allowing the generation of a limited percentage of marker-free plants (Cuellar et al., 2006; Li et al., 2007; Verweire et al., 2007). In various instances, excision events were chimeras. At present, it is not clear if this limitation reflects the functional restrictions of the transgenic use of tissue specific promoters. Although a side-by-side comparison is not available, it is reported that chemical inducible systems that control transgene excision offer higher rates of complete gene excision (Sreekala et al., 2005).

3. Chemical inducible promoters

As the activity of a gene is mainly regulated by its 5′ cis-controlling sequence, the use of promoters that respond to chemical compounds has represented a very attractive strategy for the construction of inducible gene expression systems. In theory, placing a transgene under the control of an inducible promoter will render its activity silent in the absence of the inducer and its expression regulatable by the presence of a specific signal. Ideally, with an appropriate chemical inducer, it would be possible to control transgene activity spatially, restricting the expression to particular organs or cell types; temporally, analysing the effects at defined developmental stages; and quantitatively, to study in complementation experiments the endogenous responses in a suitable window of activity. For studies of plant molecular biology, an excellent inducible expression system should have the following features: firstly, the level of expression in the absence of the inducer should be extremely low and gene activity should rise — and fall — quickly and significantly in direct correlation with the amount of inducer. Secondly, the inducer should not have any pleiotropic effect on endogenous gene expression and neither should it be toxic for plants. Thirdly, the inducer should be applicable by different means both *in vivo* and *in vitro*. All these features are probably not strictly necessary for most commercial and field applications, as important limiting factors are the cost of the inducer and its impact on the ecosystem (Table 1). Considering that it is difficult to match all these requirements, it is understandable that several plant inducible gene expression systems have been described, yet they rely upon either endogenous or exogenous elements to control transgene activity.

3.1. Plant inducible controlling elements

In plants, many, if not almost all genes respond to endogenous and exogenous stimuli, including light (Gilmartin and Chua, 1990), low and high temperatures (Kirch et al., 1997; Prandl et al., 1995), phytohormones (Farago et al., 1994), nutrients (Zhang and Forde, 1998) and other plant regulators (Hoa et al., 2002). As these signals can significantly affect endogenous gene expression and are difficult to control *in vivo*, only plant genes that respond to specific chemical compounds have been considered valuable for controlling transgene expression. Essentially, four different types of chemicals that enhance the activity of plant genes have been tested for the construction of plant inducible expression systems. They include compounds that

Table 1
Desirable characteristics of a chemical inducer for the control of transgene expression in plants.

Features required for most applications in plant biology[a]	Additional features for application in agriculture
Specific, with no pleiotropic effects	Inexpensive
Full induction below toxicity	Stable in field (non-volatile)
Efficient transport throughout the plant	Easy to get, to store and to use (exploit already existing equipment)
Rapid induction response after treatment	Low impact on agro-ecosystem (biodegradable and environmentally safe)
Appropriate for repeated on and off responses	Efficient at low concentrations and low use rates
Applicability to a variety of plant species	Suitable for short- and long-term treatments
Works in approximately dose dependent manner	Not affecting crop quality (not smelly, unpalatable, etc.)
Suitable for different methods of applications (foliar sprays, root drenches, liquid growth media and vapour)	Availability of derivatives with different properties (stability, uptake, xylematic or phloematic translocation, etc.)

[a] Some properties (*e.g.* local vs systemic activation) can be considered an advantage or a disadvantage according to specific needs. For details on the specific features of the most used chemical inducers see Moore et al. (2006).

activate genes required for Systemic Acquired Resistance (SAR), elicitors, safeners and wound signals (Gatz, 1997).

3.1.1. SAR-related activators

An important defence mechanism in plants is called the hypersensitive response, which is characterised by the rapid necrosis of the cells surrounding an invading pathogen (Durrant and Dong, 2004). The resistance mechanism also includes a long-lasting physiological immunity in uninfected tissue named Systemic Acquired Resistance. SAR is associated with the increased level of expression of several genes (collectively referred to as SAR genes), associated with the appearance of at least five families of pathogen-related (PR) proteins (Durrant and Dong, 2004). There are a number of chemically diverse molecules able to induce disease resistance in plants (Schreiber and Desveaux, 2008). Among these, chemicals referred to as SAR-inducing compounds can be used to induce PR promoters. It is long known that both salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA), when applied to leaves, induce resistance against a spectrum of pathogens and the expression of same SAR genes as tobacco mosaic virus. For these reasons these two compounds have been widely studied in both applied and basic research (Schreiber and Desveaux, 2008). A typical feature is that SA promotes expression only in the leaf tissues that have been treated, probably as result of its rapid conversion to a non-mobile glucoside (Malamy and Klessig, 1992). Hence, SA may still be useful for local induction of PR promoters. Conversely, INA induces SAR in both untreated and distal leaves. Its systemic activity is probably due to its translocation, rather than the generation of a systemic signal. While SA and INA have been shown to be potent inducers, both are phytotoxic to a degree, which has prevented their use as plant protection compounds as well as inducing chemicals for regulated expression system in plant biology (Lawton et al., 1996). Currently, as inducer of PR promoters, the choice is mainly restricted to a synthetic benzothiadiazole derivative, the benzo(1,2,3)thiadiazole-7-carbothioic acid s-methyl ester (BTH). BTH has been shown to induce the same suite of SAR genes as SA or INA at substantially higher efficacy than SA, and to act systemically (Gorlach et al., 1996). Furthermore, BTH does not lead to the accumulation of salicylic acid. These features are the most likely explanation why, at appropriate concentrations, tobacco plants treated with BTH did not show negative effects on yield or phenotype (Friedrich et al., 1996). Subsequently, BTH has also been shown to be an efficient inducer in *Arabidopsis thaliana* and other species (Lawton et al., 1996; Schreiber and Desveaux, 2008).

Among the SAR responsive genes that have been cloned, much of the work has been carried out on the *PR-1a* promoter (Gatz, 1997). This promoter is highly induced by exogenously applied chemicals with the most preferable compound being the BTH (Gorlach et al., 1996). Nevertheless, the *PR-1a* gene is expressed during seed development (Cote' et al., 1991), PR proteins accumulate in undifferentiated callus (Antowin et al., 1983) and *PR-1* transcription has been detected in regenerating tobacco (Malami et al., 1996), underlining the limitations of the applicability of endogenous elements for the control of lethal or detrimental genes.

One of the early applications of the *PR-1a* gene promoter to plant biotechnology is related to pest resistance. This promoter was used to control the expression of a ∂-endotoxin from *Bacillus thuringiensis* in tobacco (and much later in broccoli), to make the expression of the lepidopteran-specific toxin inducible by exogenous chemical application as well as by pathogen attack (Cao et al., 2006; Williams et al., 1992). The aim was that the temporal regulation of the toxin is predicted to limit the rate at which a pest population evolves towards resistance. Hence, an inducible promoter was not used to improve the desired plant phenotype (pest resistance) but to limit its possible impact of a GMO on the agro-ecosystem. However, timing, dose and cost of the treatments along with the possible pleiotropic effect on plants and the need to guarantee a good level of expression in all tissues potentially damaged by larvae, pose considerable limitations to this approach, especially if compared to the constitutive expression of *cry* genes or pesticide treatments. Furthermore, the PR1-a promoter proved also for this application to be leaky, accumulating an amount of protein that increased insect mortality in leaves that were not induced (Cao et al., 2006). Currently, other strategies are successfully used to delay the incidence of pest resistance to Bt proteins, and the most common is based on the high dose/refuge scheme (Bates et al., 2005).

3.1.2. Elicitors

Elicitors are a wide group of chemical compounds that are involved in the coordinated resistance strategy of plants against pathogens, and both organic and inorganic molecules, when exogenously applied, have been shown to act as elicitors in plant-pathogen interactions (Schreiber and Desveaux, 2008). Although an elicitor-inducible promoter based upon the bean *chalcone synthase* gene was successfully used to control the expression of the *gus* reporter gene in tobacco (Doerner et al., 1990), the fact that elicitors act upstream of the SAR response results in even more pleiotropic effects (Schmid et al., 1990), limiting the attractiveness of these compounds.

3.1.3. Safeners

Safeners are a group of chemicals that enhance a plant's tolerance to the toxic effects of herbicides. Their ability is the result of an accelerated detoxification of the herbicidal compound rather than a limitation of the uptake. The most extensively studied safener-induced genes belong to the glutathione-S transferase (GST) family and, considering their role, much of the work on these chemicals has been carried out in relation to the resistance to herbicides (Edwards et al., 2005; Farago et al., 1994; Gatz, 1997). Nonetheless, the promoter of the maize *IN2-2* gene was used to induce the expression of a *gus* gene in *Arabidopsis* (DeVeylder et al., 1997), but the translocation efficiency of the inducer benzenesulfonamide and its expected pleiotropic effects in dicotyledonous plants such *Arabidopsis* are points that have not been completely clarified (DeVeylder et al., 1997).

3.1.4. Wound signals

Wounding is able to induce the expression of various proteins at the damaged site and in addition, a set of genes is also induced systemically (Leon et al., 2001). Initially in tobacco and potato, it was demonstrated that the cis-acting element of one of these genes, encoding the potato Proteinase Inhibitor II, conferred inducibility to transgenes (Keil et al., 1989). Similarly to tissue specific promoters, it was also observed constitutive expression in some tissues and stages (Keil et al., 1989). Besides using mechanical damage, the exogenous control of wound inducible promoters can be achieved using chemical signals, which are expected to promote the endogenous expression of this class of genes. In some plant species, endogenous mobile signals that control wound-inducible genes have been identified (Leon et al., 2001). One the best characterised is the 18-aa systemin signal molecule (Howe, 2004; Schilmiller and Howe, 2005) that promotes the synthesis of jasmonic acid (JA), a key molecule required for the long-distance signal response (Stratmann, 2003). Most of our knowledge on systemin-mediated physiological functions relates to plant direct and indirect defence mechanisms against pests (Corrado et al., 2007; McGurl et al., 1994) and its role in the JA-pathway (Howe, 2004; Schilmiller and Howe, 2005). So far, studies that exploit the exogenous application of this plant signal to control transgene expression are not available, although systemin, at extremely low concentration, is a potent activator of defense gene when supplied to tomato cells or plants (Howe, 2004; Stennis et al., 1998). However, a point that should be carefully evaluated is that the use of systemin as a chemical inducer (or of a synthetic analogue) will increase the JA amount in plants over physiologic conditions, which will strongly affect diverse metabolic parameters (Pauwels et al., 2009). The use of an alternative chemical to increase plant endogenous defense such as bestatin, an aminopeptidase

inhibitor, may overcome this problem as it activates the same signalling pathway but does not lead to the accumulation of JA (Schaller et al., 1995).

In plant biotechnology, wound inducible promoters have been successfully exploited to increase pathogen resistance in absence of a chemical treatment, as many phytopathogenic fungi causes leaf lesions during their infection process (Corrado et al., 2005; Keller et al., 1999; Rizhsky and Mittler, 2001). Moreover, the wound inducible expression of an antiviral protein is also a feasible strategy for engineering virus resistance (Corrado et al., 2008). In these instances, the use of wound inducible promoter offers the advantage of confining the expression at infected sites, allowing the use of cytotoxic proteins to fight-back invading pathogens (Corrado et al., 2005; Logemann et al., 1992). Similarly, it is also possible to obtain durable and broad-spectrum resistance by inducing reactions that mimic naturally occurring defence mechanisms such as the hypersensitive cell death at infection sites (Strittmatter et al., 1995). However, while the use of wound-inducible controlling elements can have some interesting field application, its usefulness in basic plant biology is limited by the lack of systemically mobile signals that can assure a controlled and uniform activation in the whole plant (Gatz, 1997).

3.1.5. Remarks on plant controlling cis-elements

A common point of plant-based control elements is that the chemical inducer will also activate native genes. As a result, when high levels of the inducer are required, problems of phytotoxicity can frequently arise. In addition, these promoters are likely to have high level of background expression in plants and the complexity of their 5′ upstream regions make them likely to respond in a complex way to different stimuli (Zuo and Chua, 2000). For agricultural applications, the simultaneous induction of endogenous genes is not necessarily a disadvantage. An example is represented by defence genes (*e.g.*: those involved in pathogen and pest responses) that may be activated in plants alongside the transgene of interest (Breitler et al., 2001; Cao et al., 2001; Duan et al., 1996; Rushton et al., 2002; Vila et al., 2005). In all these cases, the lack of specificity may be seen as beneficial. Nevertheless, their features make these chemical inducible systems less suitable for basic research.

3.2. Heterologous elements for inducible gene expression in plants

The use of heterologous elements for the control of transgene expression in plants has the advantage that regulatory mechanisms of evolutionary distant organisms should not be related to those of higher plants. Consequently, their presence and induction should not affect endogenous gene expression. For this reason, several inducible systems have originated from components of quite diverse organisms such as viruses, bacteria, yeast, fungi, insects and mammals. Despite this obvious advantage, transferring heterologous elements into plants is not always straightforward and some complications can also arise. In a simplistic way, it is true that chemical responsive transactivator proteins and their related cis-acting sequences should work in a similar fashion in plants even if they originate from different organisms, as in nature the main features of the basal transcription machinery are well-conserved. Nonetheless, modifications of the elements of the inducible gene expression system are usually required to obtain satisfactory results. An additional constraint is that the choice of the chemical inducer is restricted to compounds that do not have any equivalent in plants, and their use should always be tested and evaluated despite the physiological difference between higher plants and other organisms.

Various plant inducible gene expression systems, based on elements from both prokaryotes and eukaryotes, have been developed, but the strategy employed is based either on the transcriptional repression/activation or the post-translational control of the protein of interest. The main difference between the two is that the transcriptional control of gene expression requires the presence of both cis and trans-acting elements (*i.e.*: an inducible/repressible promoter and a suitable transcription factor or inactivating protein) in plants, while the latter strategy only requires the expression of a fusion protein.

3.2.1. Transcriptional control of gene expression by promoter repressing/activating systems

Contrary to eukaryotes, where chromatin imposes a prevailing repressive state to many genes that are usually activated by sequence-specific DNA–protein interactions, in prokaryotes, repression of gene expression is often obtained by steric hindrance of promoter sequences by DNA-binding proteins. Because of their simplicity and wealth of knowledge, two bacterial operator–repressor systems (Lac and Tet) have been tested for the construction of plant inducible gene expression systems. Initially, attempts were made using the very well characterised *E. coli* Lac system, which is responsible for the control of the *lac* operon. In bacteria, this system provides a positive feedback loop response to lactose, which from another point of view can be seen as being an endogenous leaky system. Using IPTG as synthetic inducer, a significant relief of the transcriptional repression was observed in tobacco protoplasts (Wilde et al., 1992). More work has been carried out utilising the Tet repressor protein (TetR), which binds the tet-operator (tetO) of the tetracycline resistance gene in *E. coli* (Gatz et al., 1991). The DNA-binding ability of the TetR protein is abolished in the presence of tetracycline. Hence, in transgenic plants that constitutively express the TetR, a chimeric plant promoter containing an appropriate arrangement of tet-operators can be de-repressed by supplying significant amount of antibiotic (Gatz et al., 1992). One obstruction to the widespread use of this system was that it proved to be successful in tobacco (Faiss et al., 1997) but not in *Arabidopsis*. Other important limitations are the stability of the induction over generations, the limited half-life of the tetracycline and its limited uptake in tissue culture, which require a frequent supply of the inducer for gene activation.

The DNA-binding ability of the wild-type tet repressor has been exploited to create a tetracycline-controlled transactivator protein (tTA), by fusing the C-terminal TetR domain to the *Herpes simplex* virion protein 16 (VP16). With this strategy, the gene to be induced is under the control of a promoter containing multiple copies of the tet-operator sequences linked to a minimal 35S CaMV promoter. The tTA proteins can bind to the tetO array and stimulate its transcription in the absence of tetracycline. Tetracycline abolishes the binding of the tTA and consequently, the activity of the chimeric promoter is reduced to a very low level (Weinmann et al., 1994). While this system proved to be useful in modulating the level of expression of a GFP marker in *Arabidopsis* (Love et al., 2000) and had a lower background level in the presence of tetracycline compared to the tet-inducible system (Weinmann et al., 1994), it mirrored the limitations of the original system, as a constant amount of antibiotic is required to completely switch off gene expression. Furthermore, it displays the same problems of stability over generations as the TetR system (Gatz, 1998).

A tight control of transgene expression in higher eukaryotes is more likely to be achieved by promoter activation, rather than by repression or inactivation. In the latter strategies, it is very likely that repressor molecules have to compete with endogenous transcription factors for binding the DNA, and therefore continuous close-to-absolute occupancy of the binding sites is needed for a flawless repression. Furthermore, the requirement of an assiduous application of the chemical makes such systems less convenient to use, as illustrated by the TetR system (Zuo and Chua, 2000). For these reasons, almost all the more recent inducible gene expression systems described in plants are based on transcriptional activation. Given that in eukaryotes promoter specificity is usually the result of the quantitative interaction between cis-elements and trans-regulatory factors, the strategy is to express a transcriptional activator that, in response to a chemical compound, would induce the expression of a chimeric promoter containing the

appropriate DNA-binding sites linked to a TATA box. The elements of such activator protein are: i) a heterologous DNA-binding domain, to target the transcription factor to specific DNA sequences; ii) an activation domain, to promote the transcriptional activity; iii) a nuclear targeting signal, to direct the protein to the nucleus; iv) a chemically regulatable ligand-binding domain, which can be seen as a molecular switch and usually derives from nuclear modulators of gene expression of a distant organism. While all these elements can be provided by a multi-functional wild-type protein, fine tuning of these constituents has proved to have a direct impact on the performance of inducible gene expression systems (Bohner and Gatz, 2001; Martinez et al., 1999; Moore et al., 2006, 1998).

An example of a single eukaryotic ligand-dependent activator used for the creation of a plant inducible system is the ACE1 protein, which is a well-studied element of the copper-metallothionein regulatory mechanism in yeast. As this protein binds its specific DNA operators and promotes transcription only when complexed with copper or silver ions, it has been exploited to control the expression of various genes in plants in response to copper (McKenzie et al., 1998; Mett et al., 1993). However, the potential of this system for high level of expression and its effectiveness for homogenous induction throughout the whole plant have not yet been clarified. Furthermore, copper, besides being naturally present in soil, can lead to considerable phytotoxicity when accumulates in plant tissues following repeated treatments.

Another example is represented by the ethanol inducible gene expression system, based on the self-contained genetic system that controls the cellular response to ethanol in the fungus *Aspergillus nidulans*. This system, established upon the AlcR transcription factor and its responsive promoter *AlcA*, has been successfully used in tobacco, *Arabidopsis*, potato, oilseed rape, tomato and rice (Caddick et al., 1988; Roslan et al., 2001; Runzhi et al., 2005). As ethanol is cheap, readily available, non-toxic in moderate amount and can be easily supplied to the plants, this system is considered to have a great potential for field application, especially with the possible development of a non-volatile inducer. The alc switch also responds to several non-toxic chemicals such as acetaldehyde, acetone, threonine, ethylamine, propan-1-ol, and butan-2-ol (Runzhi et al., 2005). Alcohols that are generally used as the exogenous chemical inducer are often volatile and therefore difficult to handle in an agricultural context, as large volumes of chemical may be lost during spraying. Moreover, issues such as the specificity of the inducer, the background level of expression (especially under stress conditions) and the physiological effect of long-term ethanol induction have not been completely clarified (Salter et al., 1998). At least in some species, low concentration of ethanol has profound effects on some physiological parameters and gene expression (Camargo et al., 2007; Claassens et al., 2005). Finally, the ethanol inducible system has some detectable expression when used in plant callus and cell suspension cultures, possibly resulting from endogenous inducer produced in response to low oxygen availability (Roberts et al., 2005).

The evidence that many ligand-binding domains of nuclear receptors maintain their characteristics in fusion proteins has made it possible to create a variety of inducible transcriptional activators by domain shuffling (Picard, 1994). Nonetheless, considering that any pleiotropic effect of the application of steroids to plants has not been shown, the choice of the regulatory domain has been principally restricted to nuclear steroidal receptors such as the mammalian Glucocorticoid (GR) and the Estrogen (ER) Receptors. These proteins are activated by the presence of dexamethasone and β-estradiol, respectively.

The GR protein is not only a receptor but also a transcription factor whose nuclear localisation and DNA-binding/transcriptional regulatory function are also regulated by steroids (Picard et al., 1988). Therefore, early attempts were made with a system comprising the GR protein and a promoter containing Glucocorticoid Response Elements (GREs) linked to the 35S TATA box. However, this system proved to be functional only in transient expression assays (Schena et al., 1991) and turned out to be leaky in *Arabidopsis* (Lloyd et al., 1994). For these reasons, plant inducible systems exploit only domains of the GR protein.

The receptor domain of the rat GR has been used to produce hybrid regulatable transcription factor in combination with different elements, hence providing glucocorticoid inducibility. A first example is the GVG inducible gene expression system (Aoyama and Chua, 1997). It relies upon a transcription factor that is comprised by the hormone binding domain of the rat GR, the transcriptional activation domain of the VP16 and the DNA binding domain of the yeast Gal4 protein. Although this system has shown its potential for a number of genes (Aoyama and Chua, 1997; McNellis et al., 1998; Yoshizumi et al., 1999), subsequently some limitations have arisen. These include the possibility of inducing phenotypic abnormalities and the unintentional activation of endogenous gene expression in different plant species (Amirsadeghi et al., 2007; Kang et al., 1999). Furthermore, the stability over-generations of Gal4-mediated gene expression in plants has also been questioned (Galweiler et al., 2000; Zuo and Chua, 2000).

Other examples of systems that are based on GR domains include the LhGR and the AlcGR systems (Craft et al., 2005; Roberts et al., 2005; Samalova et al., 2005). The first is a regulatable version of the LhG4 system described above (Moore et al., 1998) and is among the most popular systems for plant biology (Moore et al., 2006). The second was constructed by fusing the GR receptor domain to the C-terminus of AlcR of the ethanol switch system.

So far, two systems based on the human ER have been developed. The first is based on a fusion between the ER and the transactivation domain of the maize C1 activator. This system proved to be able to give estradiol-dependent gene expression in stably transformed maize cells (Bruce et al., 2000). The second ER-based gene expression system was developed by fusing the DNA-binding domain of the bacterial repressor LexA, the activation domain of the VP16 and the regulatory region of the ER, which include the hormone binding site and transactivation function 2 domain. This chimeric transcription factor, named XVE, significantly increased the expression of reporter genes in tobacco and *Arabidopsis* after estradiol treatment (Zuo et al., 2000). The XVE system overcame the limitations of the GVG system (Zuo et al., 2000) and was successfully used for chemical-regulated site specific DNA excision in *Arabidopsis* (Zuo et al., 2001) Additionally, the XVE system has been reported to be deregulated in transiently transformed soybean cells, probably as a result of the presence of high amount of phyto-estrogens (Zuo et al., 2000).

A common limitation of the steroid-inducible systems is that the inducer can only be used in a controlled environment. A solution was found exploiting the ligand-binding domain of the *Heliothis virescens* Ecdysone Receptor, which is also activated by the non-steroidal ecdysone agonist RH-5992 (tebufenozide), the active principle of the Confirm 2F insecticide. For inducible expression in tobacco plants, this domain was fused to the transactivating domain of VP16 and the DNA-binding and the transactivating domains of the human GR to create a chimeric transcription factor. In this case, the uninduced level of expression of the *gus* reporter gene was carefully evaluated and reported to be around 1–5% of a 35S control (Martinez et al., 1999).

A similar system is the VGE (or its reconfigured version GVE), which consists of the VP16 activation domain, the Gal4 DNA-binding domain and the ligand-binding domain of *Choristoneura fumiferana* ecdysone receptor (EcR), in combination with a target promoter containing five copies of the Gal4 UAS (Koo et al., 2004; Padidam et al., 2003). The chimeric transcription factor is activated by methoxyfenozide, which is the active principle of Intrepid 2F and Intrepid 80 WSP insecticides. The VGE system was further developed into a two-hybrid switch. The DNA-binding and transactivation domains of the inducible transcription factor were split, and expressed by two different cassettes to produce proteins that cooperatively promote the expression of the transgene. The hybrid system offered an

increased sensibility to the inducer but a higher background expression compared to the monopartite chemical inducible transcription factor (Tavva et al., 2006).

A variation to the systems described so far is the dexamethasone-inducible and tetracycline-inactivable TGV inducible gene expression system (Bohner et al., 1999), which combines both transcriptional activation and repression. This system is based upon the TGV activator (constituted by the TetR, the hormone binding domain of the rat GR and the VP16 transcriptional activation domain) and a plant promoter in which seven copies of the tet-operator are linked to a minimal 35S promoter. While the GR domain is responsible for the activation of the chimeric transcription factor upon dexamethasone treatment, the TetR has a dual role, being able to direct the chimeric protein to the tet-operators but, at the same time, antagonising transcription in the presence of tetracycline. However, the tet-operators are believed to be prone to methylation-dependent gene silencing in plants, which limits the applicability of the system. Point mutations of the CpG and CpNpG sites in the inducible promoter were thought to solve this problem but resulted in a higher background activity (Bohner et al., 1999).

3.2.2. Post-translational activation

The hormone-binding domain (HBD) of the mammalian GR protein can be separated from other regions necessary for the transcriptional regulation of the GREs. The HBD is also known to be responsible for the assembly of the GR into an inhibitory protein complex containing Heat Shock proteins in different eukaryotes (Scherrer et al., 1993). This complex is resolved upon steroid binding, which ultimately induces the translocation of the GR protein to the nucleus (Picard and Yamamoto, 1987). Considering that such properties are conserved when the HBD is linked to other proteins (Picard et al., 1988), the HBD has been exploited to confer hormone-dependant expression on a variety of heterologous proteins in mammalian cells (Picard, 1994). Hence, this strategy relies on the constitutive expression of a chimeric protein that is post-translationally activated by dexamethasone, and this approach has also been used in plants. By fusing the maize transcription factor R to the HBD of the rat GR, it was created a transcription factor whose activity was regulated in transgenic *Arabidopsis* by the presence of steroids (Lloyd et al., 1994). Subsequently, this inducible gene expression system has been employed to control the expression of other transcription factors (Aoyama et al., 1995; Simon et al., 1996) in *Arabidopsis* and data are not available for other plant species. More recently it has also been reported that the fusion protein does not always has the expected cytoplasmic localization of in absence of dexamethasone (Brockmann et al., 2001).

4. Perspectives

4.1. Crop biotechnology

Chemically inducible systems are essential in biotechnology to control transgenes that would affect yield and quality beyond tolerability when constitutively expressed in plants. Applications are varied and include, for instance, the synchronization of flowering and fruit ripening to facilitate mechanical harvest and a more efficient post-harvest management. A promising approach that could extend the past success of classic breeding, is to express at specific phenological stages genes that alter plant architecture and resource partitioning, allowing an increase in yield without detrimental effects on other plant growing phases (Ait-ali et al., 2003; Curtis et al., 2005). Another wide area of interest is related to the fact that, as previously mentioned, the financial return for suppliers of (transgenic) crops is upheld over time if genes are present into a hybrid genotype. If the transgene of interest is placed under chemical control, thus activated only by a specific inducer protected by patent, the value of the cultivar will be also maintained for non-hybrid crops. The association proprietary chemical-transgene is at the basis of the commercial success of herbicide tolerant GMOs. However, it is difficult to foresee if this approach will be economically practicable and will not raise public concerns about the commercial strategies of biotech companies (Conway, 2000).

We hope that chemical inducible expression systems will be primarily used to increase public acceptance of GMOs, reducing some public concerns over health and environmental issues that are associated with constitutive expression.

For the expression of defence proteins against biotic stress (*e.g.*: pests and fungi), an inducible system allows the activity of a transgene to be limited in a temporal and, in appropriate applications, spatial manner. Such strategy strongly reduces negative aspects of constitutive expression such as the possible development of resistance in target organisms, the effects on non-target organisms and the unnecessary accumulation of toxic proteins in edible organs and in the environment.

Under these perspectives, chemical inducible expression systems represent an opportunity for a better resistance management (Bates et al., 2005). On the other hand, although feasible in various cases and applications (Cao et al., 2001; Corrado et al., 2008; Koo et al., 2004; Williams et al., 1992), it is certainly not universal that with an inducible expression it is always possible to match the resistance level obtained using a strong constitutive promoter. In addition, controlled systems allows also the over-expression of genes that activate broad-spectrum defence mechanisms, which may have adverse side-effects on plants when ectopically expressed (Campbell et al., 2002), or of cytotoxic protein that may promote localized cell death (Corrado et al., 2005; Logemann et al., 1992).

The presence of two insecticides among the chemical inducers available for field application offers the opportunity to combine direct and indirect plant resistance mechanisms to control pest. Under this perspective, the inducer will act as a means to directly reduce insect population and concurrently, to activate the expression of a transgene that will enhance indirect defence mechanisms of plants (*i.e.*: the ability to recruit natural enemies of pests, predators and parasitoids). This strategy will offer to reduce the amount of active principle required to control pest population by enhancing the success of controlled launch of beneficial insects in Integrated Pest Management schemes. In the future, as plants are equipped with an arsenal of defences to combat pathogen and pests attacks, it will be beneficial to use for the activation of a resistance transgene chemicals that will work with the plant's own defensive resources to generate a broader resistance response (Corrado et al., 2007).

One of the major issues of public concern regarding biotech crops has been the use of marker genes to assist the selection process of transgenic material. Even though a scientific distinction should be made between the risks associated with negative or positive selection schemes, as new technologies become available, recommendations have been made to eliminate marker genes from GM plants. So far, GM crops approved for commercial use contain a selectable marker, in the majority of cases the *npt II* gene (Miki and McHugh, 2004). It is expected that second generation GMOs will be mainly marker-free. In the High-Lysine Corn, the controlled expression of the *Corynebacterium glutamicum*-derived lysine-insensitive *dihydrodipicolinate synthase* (cDHDPS) was employed to enhance limiting amino acids in feed. The transgenic event (LY038) in the pre-launch phase was obtained by stable integration into the maize genome of a DNA fragment with the *npt II* gene cassette cloned between two loxP sequences, to allow for its subsequent removal by a Cre-*lox* recombination system. To this aim, the transgenic line was first crossed to another maize line constitutively expressing the Cre recombinase, and subsequently, the Cre cassette was removed from the hybrid by segregation. It is evident that chemical inducible systems can save years of genetic work.

An approach to obtain marker free transgenic plants is based on the chemical control of the expression of genes that are indispensable

during genetic transformation, *e.g.* those related to the hormone biosynthesis (Kunkel et al., 1999). Such approach has some functional limitations being applicable only to plants systems that undergo a regeneration process during transformation; furthermore, it requires a carefully titration of the expression level of the inducible transgene. As previously discussed, a more promising approach is the use of chemical inducible promoters to control site-specific recombinase-mediated excision of marker genes (Hare and Chua, 2002; Sreekala et al., 2005).

Finally, besides to alleviate public concerns, the inducible removal of marker genes is a convenient option to allow re-trasformation process and it is also useful to reduce the homology-dependant silencing risks in gene stacking strategies (Halpin, 2005).

4.2. Inducible promoters and bioreactors

Simple inducers of metabolic processes are widely used in microbial systems (Sanchez and Demain, 2002). However, the possibilities of regulating the environment of auxotrophs in field are much more limited, which strongly restricts the number of potentially useful compounds to induce gene expression. Transfer to special growth conditions for gene induction is not practicable in agriculture, where conditions are optimised to maximise plant yield. Starvation for a particular nutrient or the addiction of chemicals that induces phytotoxicity may be acceptable only in particular situations (Schreiber and Desveaux, 2008). Nonetheless, such constraint is not valid in bioreactors of photoautotrophic organisms, in which a whole range of chemical compounds can be used.

Although large-scale production of plant secondary metabolites in bioreactors is commercially feasible (Makkar et al., 2007), costs must be significantly reduced to establish plant cell culture as a competitive alternative of microbial systems for production of recombinant proteins (Demain and Vaishnav, 2009). Increase in productivity is expected to rely especially on advances in plant molecular biology and innovative engineering solutions, which should allow higher yields and reduced manufacturing costs, respectively (Zhong, 2001). Although there are several areas of improvements, chemical inducible systems will also play an important role, considering that proteins are direct gene products. When a constitutive promoter is used to control transgene expression, the production of a recombinant protein in a cell culture system is largely dependent on the cell growth phase. Plant cells will continue to accumulate proteins also following the entrance into the stationary phase, but this is usually accompanied with increased proteolytic activities and more difficulties for protein purification (Zhong, 2001). These are the main reasons why the recombinant protein level tends to descend during late stationary phase (when the 35S RNA promoter is used), limiting the overall yield and usability of the cell culture. Inducible promoters allow the activation of the transgene when the culture reaches a suitable biomass in the late exponential growth phase, with the possibility to uncouple the growth phase from the protein production. To this goal, one of the most cost-effective strategy is represented by promoters activated by sugar starvation such as the rice α-amylase gene promoter (Trexler et al., 2002).

The confined nature of the cell culture allows the use of a huge variety of promoters controlled by either chemicals or physical parameters such as light and temperature (Nara et al., 2000; Peebles et al., 2007; Uozumi et al., 1994; Yoshida et al., 1995). Depending on the nature of the inducer and of the recombinant protein, repeated treatments may be possible and desirable. For instance, recurring inductions can be used to control cell-cycle genes to transiently increase cell growth rate prior the induction of the recombinant protein (Koroleva et al., 2004). Finally, an unexplored opportunity is the accumulation of a protein (or more generally of bioproducts such as biopolymers and bioplastics) and its subsequent enzymatic modification following the controlled sequential activation of different transgenes in the same cell culture (Mooney, 2009).

5. Concluding remarks

This review has analysed a number of chemical inducible gene expression systems for plants and has focussed on their applications in biotechnology, molecular breeding and agriculture. Nonetheless, besides the scientific considerations we discussed, the final evaluation for commercial applications should also include regulatory and financial issues. A significant number of plant inducible systems have been developed and many of them reproduce or exploit similar strategies that have already been tested in bacteria, yeasts, *Drosophila* or mammalian cells. All these systems proved to be effective in inducing the expression of reporter genes yet, to date, none of them is of universal application. One reason may be that an ideal system (*i.e.*: highly inducible, tightly controlled, stable over generations and effective in different species) is difficult to obtain and probably, according to specific necessities and applications, one system may be more useful than others. While the choice for plant biology is wide, very few chemical inducible systems are practical for field application. Systems based on plant controlling elements have some intrinsic limitations (*e.g.*: leaky expression, perturbation of plant function, activation of endogenous genes and poor/non uniform uptake or translocation of the inducer) that make them useful for specific purposes and conditions. Broader applications can be pursued using systems based on chimeric promoters. Among these, only the Alc, the VGE/GVE and the EcRVG can be proposed as commercially feasible options, since they exploit chemical inducers that could be used in fields. However, the real convenience of the ethanol system for agriculture has also been questioned (Vreugdenhil et al., 2006). While the Alc system relies on a single multifunctional trans-activating protein, the other systems are based on a chimeric protein in which, for instance, methoxyfenoside inducibility is due to the spruce budworm EcR receptor. This regulatory element can be incorporated into any existing or proprietary transcription factor to obtain chemical inducibility (Moore et al., 2006; Tavva et al., 2007), allowing the development of more complex or specific systems. On the other hand, local induction by foliar spray seems to be possible only for the Alc system, although ethanol is efficiently transported throughout the plant (Moore et al., 2006).

It is likely that the popularity and the abundance of chemical regulated expression systems that are unsuitable for field application has limited the diffusion of this approach in plant biotechnology (Fig. 1). An agriculturally suitable chemical inducible system should rely on compounds that may be applied without causing unacceptable levels of soil damage, phytotoxicity in the crop and pollution in the environment (Table 1).

Several systems rely on chemical-responsive transcription factors of non-plant biological systems mainly because elements from other organisms provide a tight and specific control of transgene expression, a crucial feature for functional studies. In addition, the wealth of knowledge about classic biological model systems has strongly influenced the early development of plant regulatable systems. Plant functional genomics is bridging the gap and a number of scientific tools, available for both model and crop species, can provide information for the development of new inducible systems based on plant chemically regulatable elements. Reversing the argument that led to the construction of heterologous expression systems, chimeric systems that exploit plant-derived elements are expected to rely on chemicals that should have a negligible effect on mammals and more generally on animals. Nonetheless, design strategies for the development of synthetic promoters and their cognate DNA binding proteins are still in their infancy (Venter, 2007). It is more likely that in the near future, progresses for plant biotechnology will derive from functional and molecular studies of the effects of agro-chemicals. Many of these compounds, which have been already registered for use in agriculture, have characteristics of an ideal chemical inducer for field application. They are relatively cheap and easy to use, approved as environmentally

compatible, and highly efficient at low concentrations and low use rates. In addition, a range of derivatives with different properties is available, including agonists or antagonists, compounds that move systemically or are confined to the site of application, and variants with different stability in plants and in the agro-ecosystem. By selecting combinations of inducing compounds with different rates of conversion, it will be possible to sustain gene expression over a longer period with a single treatment, instead of using repeated applications. On the other hand, agro-chemicals, should not be considered totally harmless as they are designed to affect target organisms or plant physiology. The feasibility of this approach has been recently demonstrated for glyphosate, one of the most common broad-spectrum herbicide. In soybean, three cDNAs were found to have a convenient time- and dose-dependent expression patterns in relation to glyphosate. This suggests that it may be possible to identify cis-controlling elements and possibly chemical inducible transcriptional activators for the construction of an herbicide inducible system (Yu et al., 2007). Since the constitutive expression of glyphosate-resistant genes may result in some detrimental effects on host plants (Pline-Srnic, 2005), an obvious application of this yet-to-come inducible system will be to control genes that confer herbicide tolerance.

An environmentally safer option will be the use of inactive chemical compounds that are hydrolysed in the target plant either chemically, or enzymatically by a naturally occurring enzyme or by an enzyme introduced by genetic engineering into the plant. In this case, the expression of the enzyme ultimately necessary for the activation of transgene of interest could be controlled by a positive feedback loop, placing its coding sequence under the control of the (leaky) chemically inducible promoter.

In conclusion, while chemical inducible systems have been widely adapted in plant biology, their use in plant biotechnology is comparatively more limited. It is likely that this reflects the reduced number of systems suitable for field application, in conjunction with the fact the commercially successful GMOs encode genes for resistance to biotic stress and/or herbicide. Further developments are still necessary to expand the number of inducers that are appropriate for agriculture. Plant "omics" should provide essential information to guide the construction of new biotechnology-oriented systems, since the most recent advances indicate that regulated gene expression systems will play an increasingly important role in the second generation of GMOs, in bioreactors and possibly for a better management of resistance genes against biotic stress.

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