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Genetic Transformation in the Breeding of Flower Bulbs

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ABSTRACT

Ornamental geophytes are used for the production of cut-flowers, potted flowering plants or in gardening (collectively known as flower bulbs). Most flower-bulb cultivars have been produced by cross hybridization and mutation breeding and are propagated vegetatively. Biotechnological techniques have been used to breed and propagate these plants. Plant breeders use *in vitro* techniques, such as cut-style in vitro fertilization, embryo rescue, ovary-slice culture and ovule culture, to overcome pre- or post fertilization compatibility barriers and generate interspecific hybrids. Recently, biotechnological tools such as molecular markers and genetic engineering have also been introduced. Genetic transformation may be defined as the utilization of isolated recombinant DNA based technology to aid the effective incorporation of a limited number of valuable traits (that are not available in the original plant genome or in closely related species) into improved cultivars lacking such traits. Transformation techniques supplement the other methods available to plant breeders and are especially valuable for clonally propagated crops, such as flower bulbs. Flower bulbs have been transformed using both *Agrobacterium*-mediated and microprojectile-acceleration methods. In both systems, the success of the transformation depends upon the successful assembly of several key components and the calibration of the entire system. One component is the availability of a genetic construct carrying target genes under the control of appropriate promoters. A second component is the target organ or tissue, which must be competent for genetic transformation. That is, it must be capable of accepting the foreign DNA, into the genome of its own cells, expressing the genes and maintaining the ability to regenerate into plants. Introduced genes in many agricultural crops include those that confer resistance to biotic or abiotic stresses, as well as genes that alter plant phenotype (e.g., flower color). Although transformation systems for many flower-bulb crops are available, few attempts to produce genetically engineered flower bulbs with commercially valuable traits have been successful and, to date, none have resulted in a registered cultivar. In order to be commercially viable, any genetically engineered flower bulb cultivar would contain mostly proprietary technology covered by freedom-to-operate agreements. Marker-free technology is needed to ease the risk-assessment process and to address public concerns.

Keywords: biotechnology, genetic transformation, molecular breeding, ornamental geophytes, tissue culture
Abbreviations: BYMV, *Bean yellow mosaic virus*; **CMV**, *Cucumber mosaic virus*; **CaMV**, *Cauliflower mosaic virus*; **CP**, coat-protein; **GM**, genetically modified; **GFP**, green fluorescent protein; **GUS**, β-glucuronidase; **HPLC**, high-performance liquid chromatography; **hpt**, hygromycin phosphotransferase; **nptII**, neomycin phosphotransferase; **OrMV**, *Ornithogalum mosaic virus*; **PAT**, phosphinothricin acetyltransferase; **TPN1**, tachyplesin 1; **UBQ3**, *Arabidopsis* ubiquitin

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INTRODUCTION

Biotechnology may be defined as any technological application that uses biological systems, living organisms or derivatives thereof to construct or modify processes for a specific use. This definition is broad enough to include practically any technical innovation used in the past, presently employed or developed for future use in research, development or commerce. Biotechnological techniques have long been used in the horticultural industry, but the use of the term itself has changed over the years. From the 1960's through the 1980's, this term was mainly used to

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describe the handling of plant tissue *in vitro*. However, during the last two decades, it has generally been used in the context of the application of molecular techniques and the use of molecular markers.

Ornamental geophytes used for the production of cut-flower crops, potted flowering plants or in gardening are collectively referred to as flower-bulbs. This term is broadly used in connection with all ornamental plants that have subterranean storage organs, the main function of which is to store food reserves, moisture and nutrients to ensure the long-term survival of the species (De Hertogh and Le Nard 1993a). However, these storage organs are morphologically and functionally very diverse. They include true bulbs, corms, tubers, tuberous roots, rhizomes and enlarged hypocotyls. Most flower-bulb species are monocots, yet some are dicots, and they belong to many different plant families and a variety of genera. One would expect this enormous diversity to manifest itself in all aspects of plant growth and development, with implications for the intended uses of the different species, forcing, propagation, breeding objectives and breeding techniques. From an economic perspective, we should acknowledge the fact that 90% of world production of flower bulbs is accounted for by only six genera, namely, *Tulipa*, *Lilium*, *Gladiolus*, *Narcissus*, *Iris* and *Hyacinthus* (De Hertogh and Le Nard 1993b). The dominant commercial positions held by these genera are also reflected in the relative volume of research involving these genera, including breeding efforts for the production of new cultivars.

Genetic transformation may be defined as the utilization of isolated recombinant DNA based technology to aid the effective incorporation of a limited number of valuable traits (that are not available in the original plant genome or in closely related species) into cultivars lacking such traits, to produce improved cultivars. The development of *in vitro* techniques proved to be the prerequisite and enabling force for molecular breeding through genetic transformation. These techniques are not intended to replace conventional breeding techniques, but rather to complement them.

The success of a transformation depends upon the successful assembly of several key components and the calibration of the entire system. One component is the availability of a genetic construct carrying a target gene, a reporter gene and an appropriate selectable marker, all under the control of appropriate promoters. A second component of the system is the target organ or tissue, which must be competent for genetic transformation. That is, it must be capable of expressing the introduced genes and maintain the ability to regenerate into plants. Another key element is the gene-delivery system. Initial attempts to transform a plant tissue usually result in many cells that express the transgene(s) transiently. Only in a small fraction of these cells the transgene is actually integrated into the genome and stably expressed. For a reliable and reproducible method, one has to start with a very efficient gene-delivery system that will allow the selection of a sufficient number of stable transformants. Furthermore, since transformation is a single-cell event, it is imperative that the transformed cells be provided with conditions that allow them to develop into shoots, either through direct organogenesis from the transformed tissue or through somatic embryogenesis.

GENETIC TRANSFORMATION OF FLOWER-BULB SPECIES

Gene-delivery systems

Several methods have been used to deliver foreign genes into plant genomes. Of these, the most commonly used are *Agrobacterium*-mediated and biolistic-mediated transformation processes. *Agrobacterium*-mediated transformation has been the favored method, since it offers advantages such as the defined integration of the transgene, low copy numbers and preferential integration to transcriptionally active regions of the genome (Zuker *et al*. 1998; Gelvin 2003 and references cited therein; Hoshi *et al*. 2004).

Monocotyledons have generally been thought to be recalcitrant to infection and subsequent transformation by *Agrobacterium*. Most flower-bulb species are monocots (De Hertogh and Le Nard 1993a). For these species, the preferred method, or rather the default option, for the introduction of foreign genes has been direct transformation through particle bombardment (Sanford 1988; Finer *et al*. 1992; Vain *et al*. 1995). However, numerous recent reports have challenged this approach.

1. Agrobacterium-mediated transformation of flower bulbs

Paperwhite narcissus (*Narcissus papyraceus*) was, to our knowledge, the first flower-bulb species in which *Agrobacterium*-mediated transformation was confirmed (Hooykaas-Van Slogteren *et al*. 1984). Wild-type nopaline and octopine strains of *Agrobacterium tumefaciens* were used to induce tumors from which nopaline and octopine, respectively, were extracted. Graves and Goldman (1987) reported similar results in *Gladiolus*, in which persistent expression of T-DNA-encoded genes was detected after the infection of corm disks. That study demonstrated that *A. tumefaciens* adheres to these plant cells in the same manner it adheres to those of dicotyledonous plants (Graves *et al*. 1988). Kamo (1997a) also demonstrated *Agrobacterium*-mediated transformation based upon opine synthesis and was able to detect very strong, transient β-glucuronidase (GUS) expression in dark-grown leaves and callus tissue.

Yu *et al*. (1988) inoculated *Hemerocallis citrina* (daylily) and *Lycoris radiata* with *A. tumefaciens*. Both species reacted by developing swellings in which nopaline was detected, although no tumors were produced. Conner and Dommisse (1992) infected several monocot species, including the flower-bulb species *Nerine bowdenii* and *Zantedeschia aethiopica*, with *A. tumefaciens* and *A. rhizogenes*. In their paper, they reported on the development of tumors and described how they extracted *A. tumefaciens* opines from these tumors. Similarly, Cohen and Meredith (1992) showed that *A. tumefaciens* C58 could infect and transform *Lilium* and that such infection was also strain-dependent. This report was later confirmed by Langeveld *et al*. (1995), who were able to demonstrate the expression of the GUS reporter gene in transformed lily tissue, but no plants were regenerated in their study.

Similarly, Wilmink *et al*. (1992, 1995a) reported on *Agrobacterium*-mediated transient GUS expression in stem explants of several tulip hybrids and several botanical species, even though these plants lacked the virulence-inducing substances that are essential for *Agrobacterium* infection. *A. rhizogenes* was found to be more efficient than *A. tumefaciens* and no difference was found in the numbers of transient blue spots produced following *Agrobacterium*-mediated infection as compared to transformation by the particle bombardment method, except for the botanical species that showed little reaction after particle bombardment. The lack of a reliable *in vitro* regeneration system for tulips seems to be an obstacle to the utilization of the system. Only a single confirmed transgenic tulip plant actually reached maturity, seven years following the transformation attempt (Van Tuyl and van Creij 2006).

These early reports were followed by numerous publications describing successful *Agrobacterium*-mediated transformations and the subsequent generation of transgenic flower bulbs. The genus *Lilium* (species and interspecific hybrids) has been the subject of the largest number of reports (Mercuri *et al*. 2003; Hoshi *et al*. 2004, 2005; Li *et al*. 2008; Ogaki *et al*. 2008; Thao *et al*. 2008; Ku and Tsay 2009; Azadi *et al*. 2010a, 2010b; Núñez de Cáceres *et al*. 2011). Other flower-bulb species in which *Agrobacterium* infection and subsequent transformation have been confirmed include *Caladium bicolor* (Li and Wu 1990; Li *et al*. 2005), *Iris germanica* (Jeknic *et al*. 1999), *Agapanthus praecox* (Suzuki *et al*. 2001; Suzuki and Nakano 2002a;

Mori *et al*. 2007), *Muscari armeniacum* (Suzuki and Nakano 2002a, 2002b; Suzuki *et al*. 2005), *Narcissus pseudonarcissus* (Sage and Hammatt 2002), *Alstroemeria* (Kim *et al*. 2002; Akutsu *et al*. 2004; Kim *et al*. 2007; Hoshino 2008; Hoshino *et al*. 2008), *Tricyrtis hirta* (Adachi *et al*. 2005; Nakano *et al*. 2007; Mori *et al*. 2008), *Hyacinthus orientalis* (Popowich *et al*. 2007), *Narcissus tazetta* var. *chinensis* (Lu *et al*. 2007), *Ornithogalum* (Van Emmenes *et al*. 2008) and *Zantedeschia elliottiana* (Yip *et al*. 2007).

All the above-mentioned geophytes are monocot species. It appears obvious that the notion that monocotyledonous plants are insensitive to *Agrobacterium* infection and, therefore, cannot be transformed using the *Agrobacterium*-mediated system is no longer valid. Given the right biovars or strains, the appropriate co-cultivation conditions and a competent target tissue, the *Agrobacterium*-assisted transformation of any monocot flower bulb is in principle possible.

Dicotyledonous flower bulbs have also been transformed using *Agrobacterium*. There have been reports of the transformation of *Cyclamen persicum* using etiolated petiole segments (Aida *et al*. 1999) or etiolated hypocotyls (Boase *et al*. 2002). Long-term expression of the GUS reporter gene, including meiotic stability, has been reported and reports have also confirmed that the offspring of these transformed plants inherit the transgene (see references cited by Boase *et al*. 2002). *Begonia tuberhybrida* was transformed with *A. tumefaciens* harboring *rol* genes from *A. rhizogenes* (Kiyokawa *et al*. 1996). Giri *et al*. (1997) transformed *Aconitum heterophyllum* with *A. rhizogenes*, which induced a "hairy root" syndrome in embryogenic callus. They detected the presence of mannopine in extracts of some transgenic roots and this presence was found to be dependent on the bacterial strain used.

2. Particle bombardment-mediated transformation of flower-bulb species

Direct transformation based on the acceleration of gold or tungsten microprojectiles carrying the genetic constructs into plant cells (also known as particle bombardment, particle acceleration or the gene-gun technique) has also been used successfully in flower-bulb species, as noted above. The main advantage of this method is that it is not specific to any particular plant species or cultivar. Early reports in this area described the use of particle bombardment to study the competence of explants or tissue for accepting foreign genes and/or to evaluate the efficiency of gene constructs in driving transient expression in the plant cells or in order to optimize the gene-gun system (e.g., Wilmink *et al*. (1992) in tulip, Nishihara *et al*. (1993) in lily, Wilmink *et al*. (1995b) in tulip and lily, Kamo *et al*. (1995a, 1995b) in gladiolus, Tsuchiya *et al*. (1996) in lily, Tribulato *et al*. (1997) in lily, van Schaik *et al*. (2000) in *Alstroemeria*, Tang *et al*. (2004) in crocus). Many others have been able to obtain gene insertion, stable integration into the plant genome and generation of transgenic plants through the use of this technique.

The main goal of all of these studies was the calibration of the transformation system for important plant species, for the insertion of valuable traits into the species genomes. As in the case of *Agrobacterium*-mediated transformation, *Lilium* has been the genus examined in the largest number of published reports. Watad *et al.* (1998) were the first to report the successful microprojectile bombardment-mediated transformation of *Lilium* and the recovery of transgenic plantlets. This work paved the way for several successful attempts to transform lily plants (Lipsky *et al*. 2002; Irifune *et al*. 2003; Ahn *et al.* 2004; Cohen *et al*. 2004; Benedito *et al*. 2005; Kamo and Han 2008). The transformation of the genus *Lilium* was reviewed by Cohen (2011). There have also been reports of successful biolistic transformation and subsequent regeneration of transgenic *Gladiolus* plants using suspension cells and callus tissue (Kamo *et al*. 1995b) or cormels (Kamo *et al*. 1995a).

Lin *et al*. (2000) reported the successful transformation of *Alstroemeria* following the bombardment of embryonic callus with high regeneration potential (also reviewed by Hoshino 2008). Daylily (*Hemerocallis*), one of the most important perennial geophytes in the American nursery industry, was transformed by Aziz *et al.* (2003). *Ornithogalum*, another flower-bulb genus with increasing importance in the flower industry, was transformed using the gene-gun technique. De Villiers *et al*. (2000) reported the transformation of a selected *Ornithogalum thyrsoides* x *O. dubium* hybrid. The orange-flowered *O. dubium* was also transformed using a slightly modified system with higher efficiency (Cohen *et al*. 2004).

Genetic constructs for transformation

A crucial component of any successful attempt to transform a plant is the genetic construct carried into the plant cells by the gene-delivery system. This genetic construct is generally comprised of three key components: an appropriate selectable marker, a reporter gene and a target gene. The first two are needed for the development or improvement of transformation protocols. The target gene is needed for the introduction of the desired phenotype.

1. The selectable marker

The selectable marker is a crucial component of the genetic construct. It is used to differentiate between transgenic and non-transgenic cells in the target tissue, while allowing the survival of the transgenic cells and the subsequent regeneration of transgenic shoots. The choice of the selectable marker is crucial (coupled with the selection scheme). Its activity may affect not only the possibility of regenerants arising under selection, but also the number of escapes, as well as the formation of chimeric tissues and their frequency (Zuker *et al*. 1998). The choice of the selective agent is often species- or even cultivar-dependent and relies on the calibration of proper dose-response curves for the different stages of plant development. A preferable selective agent (and preferable concentration) is one that totally inhibits the growth of non-transgenic cells without causing cell death, which would lead to the secretion of unspecified oxidative products into the surrounding culture medium. Such secretion usually causes slow down in development and death of otherwise actively growing cells (pers. obs.).

The list of selectable genes that have been used in flower-bulb transformation is by no means long. It mostly includes genes that confer resistance to an herbicide and genes that detoxify antibiotics. The selectable gene from the first group most commonly used in bulb transformation studies is the *bar* gene, which encodes for the enzyme phosphinothricin acetyltransferase (PAT). This enzyme detoxifies phosphinothricin (glufosinate-ammonium), the active ingredient in the herbicide Basta. It has been used successfully in lilies (Watad *et al*. 1998; Lipsky *et al*. 2002; Irifune *et al*. 2003; Ahn *et al*. 2004; Benedito *et al*. 2005; Kamo and Han 2008; Thao *et al*. 2008), gladioli (Kamo 1997b; Kamo *et al*. 1995a, 1995b, 2000a, 2000b, 2005, 2010), *Ornithogalum* (De Villiers 2000), *Alstroemeria* (Lin *et al*. 2000) and daylily (Aziz *et al*. 2003). The use of this herbicide as a selective agent may be problematic. The distance between the range of concentrations that allow 'escapes', the range that allows the development of chimeras and the levels that completely eliminate any regenerants is very limited. In *Ornithogalum dubium*, for instance, a concentration of 1 mg/l in the growing medium is less than the effective selective concentration and causes a minor slow down in the regeneration of non-transformed plants. A concentration of 2 mg/l, on the other hand, may be lethal to both transformed and non-transformed cells and the death of these cells leads to the secretion of oxidative material into the culture medium. When an herbicide concentration of 1.5 mg/l is used, the surviving plants are chimeric and remain in that state for many months (Cohen, unpublished data and

pers. obs.).

The second group of selectable markers includes antibiotic-resistance genes, usually of bacterial origin, that are expressed under the direction of plant promoters. The best known of the selective antibiotics used in plant transformation are members of the aminoglycoside family: kanamycin, gentamycin, G418, neomycin, paromomycin and hygromycin (Wilmink and Dons 1993). These antibiotics inhibit protein synthesis in plant cells. Kanamycin, gentamycin, geneticin (G418), neomycin and paromomycin bind the 30S ribosomal subunit and inhibit the initiation of translation and, consequently, protein synthesis. Hygromycin binds the ribosomal site of the elongation factor EF-2, inhibiting chain elongation. The properties of these antibiotics, their mode of action and their inactivating enzymes have been reviewed by Nap *et al*. (1992) and Wilmink and Dons (1993).

Kanamycin and hygromycin are commonly used as selective agents in transformation experiments in which genetic constructs carry the genes that detoxify these antibiotics. The selective marker *nptII*, which encodes for the enzyme that detoxifies kanamycin (neomycin phosphotransferase) has been used for the selection of transgenic *Lilium* plants (Mercuri *et al*. 2003; Cohen *et al*. 2004; Li *et al*. 2008; Núñez de Cáceres *et al*. 2011) and in *Begonia tuberhybrida* (Kiyokawa *et al*. 1996), *Iris germanica* (Jeknic *et al*. 1999), for which geneticin (a gentamicin-derivative) was also used; *Cyclamen* (Aida *et al*. 1999; Boase *et al*. 2002, 2010); *Agapanthus* (Suzuki *et al*. 2001; Suzuki and Nakano 2002b); *Alstroemeria* (Akutsu *et al*. 2004); *Crocus* (Tang *et al*. 2004); *Caladium* (Li *et al*. 2005); *Tricyrtis* (Adachi *et al*. 2005; Mori *et al*. 2008); *Zantedeschia* (Yip *et al*. 2007); *Hyacinthus* (Popowich *et al*. 2007) and *Ornithogalum* (Cohen *et al.* 2004, 2005; Van Emmenes 2008; Cohen 2011; Cohen *et al.* 2011).

The *hpt* gene, which encodes for hygromycin phosphotransferase and allows for the selection for hygromycin resistance, has also been widely used, sometimes in conjunction with kanamycin, in transformation attempts in *Lilium* (Hoshi *et al*. 2004; Ogaki *et al*. 2008; Azadi *et al*. 2010a, 2010b), *Iris* (Jeknic *et al*. 1999), *Cyclamen* (Aida *et al*. 1999), *Agapanthus* (Suzuki *et al*. 2001), *Alstroemeria* (Akutsu *et al*. 2004; Hoshino *et al*. 2008), *Muscari* (Suzuki and Nakano 2002a, 2002b), *Tricyrtis* (Adachi *et al*. 2005) and *Narcissus* (Lu *et al*. 2007).

2. The reporter gene

Another component of the genetic construct is the reporter gene. The presence of this gene in the genetic construct is not crucial to the transformation process or its outcome, yet its expression provides the researcher with a convenient tool for visualizing the initial success of the transformation system and any changes that occur in the plant tissue during the process. The β-GUS gene is widely used as a reporter gene in transformation studies of many crops, including flower bulbs. The GUS histochemical assay (Jefferson 1987) was initially used for the study of transient gene expression in certain tissues in many plant species, including bulb-crop genera, such as *Lilium* (Nishihara *et al*. 1993; Langeveld *et al*. 1995; Miyoshi *et al.* 1995; Wilmink *et al*. 1995b; Tsuchiya *et al*. 1996; Tribulato *et al.* 1997; Suzuki *et al*. 1998; Suzuki and Nakano 2002b), *Gladiolus* (Kamo *et al*. 1995a, 1995b; Chauvin *et al*. 1997; Kamo *et al*. 1997b), *Tulipa* (Wilmink *et al*. 1992; Chauvin *et al*. 1997), *Alstroemeria* (van Schaik *et al.* 2000) and *Crocus* (Tang *et al*. 2004) among others. This assay has also been used to monitor the entire transformation and regeneration process, including the calibration of the gene-delivery system, verification of the ability of the genetic construct to transfer genes into the plant tissue, optimization of the culture conditions (Tsuchiya *et al*. 1996; Ogaki *et al*. 2008; Azadi *et al*. 2010a) and evaluations of the efficacy of alternative promoters (Van der Leede-Plegt *et al*. 1992; Wilmink *et al*. 1995b; Kamo and Blowers 1999; Kamo *et al*. 2000a; Kamo 2003), transformation efficiency, tissue competence (Cohen *et al*. 2004), stable transgene integration and plant regeneration. In fact, almost all of the attempts to transform flower-bulb species have involved the use of GUS as a reporter gene, with the very few exceptions mentioned elsewhere in this review.

Despite being widely used, the GUS reporter gene is somewhat inconvenient, as its use requires a tissue-destructive assay. In order to monitor the progress of the transformation process, plant tissue has to be sacrificed periodically. A non-destructive alternative method based on the use of green fluorescent protein (GFP), which can be viewed *in vivo* under a UV microscope, allows for non-destructive, real-time monitoring of the plant tissue throughout the entire transformation process (Elliott *et al*. 1999; Wenck *et al*. 2003). However, some plant tissues show auto-fluorescence in green fluorescent colors under UV illumination, making the use of GFP in such cases somewhat problematic (pers. obs.). In such cases, the use of red fluorescent protein (RFP) should be a better choice for future studies. Sage and Hammatt (2002) reported on the use of GFP in transformation studies of daffodils, but they provided few details. Ku and Tsay (2009) recently used GFP to monitor transformation in both *L. longiflorum* and *L. formosanum*.

Another non-destructive reporter gene is the firefly luciferase (*luc*) gene that was used in *Alstroemeria* by Lin *et al*. (2000). As in the case of GFP, luciferase activity can be checked periodically throughout the entire transformation process, using a luminometer, without damaging the culture. However, this method is labor-intensive, extra care should be employed to avoid dehydration of the plants, and the necessary equipment is expensive, making the use of luciferase as an alternative reporter gene far less attractive.

3. The target gene

The target gene codes for the trait that is the target of the transformation. Chandler and Tanaka (2007) suggested classifying introduced traits as producer traits or consumer traits, based on whether the genetic modification primarily benefits the producer or the consumer. Producer traits include insect resistance, virus resistance, other disease resistance and manipulation of hormonal regulation. On the other hand, flower color, flower fragrance, flower shape and post-harvest quality (product longevity) may be considered consumer traits. Some traits may be of significant interest to both growers and consumers. Genes of commercial interest have been introduced into numerous ornamental crops (listed in Chandler and Lu 2005; Chandler and Tanaka 2007).

Few of the transformation studies conducted in flower-bulb species have actually involved attempts to introduce target genes. These studies and the included target genes are listed in **Table 1**. These studies may be classified according to the various goals of the transformation attempts. Some studies have attempted to introduce genes that confer resistance to bacterial or fungal diseases, both of which cause considerable losses in vegetatively propagated flower-bulb crops.

3.1. Transformation for resistance to biotic and abiotic stress

Kamo (1997a) attempted to confer resistance to *Bean yellow mosaic virus* (BYMV) in *Gladiolus* by transforming it with a BYMV coat protein. In this study, stable long-term expression of the target gene was detected. In a later report, Kamo *et al*. (2005) reported the transformation of gladioli with BYMV coat protein gene, in either its sense (CP) or antisense (AS) orientation. Infection was found to be significantly delayed in transgenic plants containing the viral genes in either orientation. These researchers challenged *Gladiolus* plants transformed with constructs containing *Cucumber mosaic virus* (CMV) subgroup I coat protein, subgroup II coat protein, CMV replicase and a combination of genes coding for the two subgroups, as well plants trans-

Table 1 Reports of attempts to incorporate specific novel target genes into different flower-bulb species and cultivars.

Species	Transformation method	Target gene	Trait	Reference
Begonia tuberhybrida	*A. tumefaciens*	*rol A,B,C*	Altered phenotype (dwarf growth habit, dark-green leaves, wrinkled leaves and flowers, delayed flowering)	Kiyokawa *et al*. 1996
Caladium bicolor	*A. tumefaciens*	Maize anthocyanin regulatory gene *(Lc)*	Enhanced anthocyanin accumulation in roots, leaves and stems	Li *et al*. 2005
Cyclamen persicum	*A. tumefaciens*	Chalcone reductase	Pale yellow flower buds	Mizukami *et al*. 2004
Cyclamen persicum	*A. tumefaciens*	Manganese superoxide dismutase (MnSOD)	Resistance to heat stress	Chen *et al*. 2008
Cyclamen persicum	*A. tumefaciens*	Flavonoid 3'5'-hydroxylase-antisense	Modification of flower color, decrease in total anthocyanin content	Boase *et al*. 2010
Gladiolus grandiflorus	Particle bombardment	BYMV- coat protein (sense or antisense)	Enhanced resistance to *Bean yellow mosaic virus*	Kamo 1997b; Kamo *et al*. 2005
Gladiolus grandiflorus	Particle bombardment	CMV-coat protein, CMV replicase	Resistance to *Cucumber mosaic virus*	Kamo *et al*. 2010
Gladiolus grandiflorus	Particle bombardment	Single-chain variable fragment antibodies (scFv) to CMV	Resistance to *Cucumber mosaic virus*	Kamo *et al*. 2012
Hyacinthus orientalis	*A. tumefaciens*	*Thaumatin II*	Resistance to *Botrytis* and *Fusarium*	Popowich *et al*. 2007
Lilium sp.	*A. tumefaciens*	Jasmonate and ethylene-responsive factor 3 protein (*JERF*3)	Enhanced salt tolerance	Yang *et al*. 2007
Lilium formosanum	*A. tumefaciens*	Flavonoid synthesis genes: *DFR*, *F3H*, *CHI*	Altered flavonoid synthesis	Ku and Tsay 2009
Lilium longiflorum	Particle bombardment	CMV-defective replicase	Intended to confer resistance to viral infection	Lipsky *et al*. 2002
Lilium longiflorum	*A. tumefaciens*	*rol A,B,C*	Altered phenotype (dwarf growth habit, short leaves, fewer and smaller flowers, dense roots)	Mercuri *et al*. 2003
Lilium longiflorum	*A. tumefaciens*	Pokeweed antiviral protein(PAP)	Expression of antiviral protein, particularly in the leaves	Wang *et al.* 2005
Lilium longiflorum X *L. formosanum*	*A. tumefaciens*	*Zm401*- a maize pollen-specific gene	Aimed to produce pollenless lilies (there have been no reports on the flowers of these plants)	Li *et al*. 2008
Lilium longiflorum X *L. formosanum*	*A. tumefaciens*	Carotenoid biosynthesis key gene cassette: *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, *crtW*, *idi*	Intended to introduce novel orange flower colors to white-flower lily	Azadi *et al.* 2010b
Lilium longiflorum	Particle bombardment	*rol B*	Pollen-less flowers, smaller flowers	Cohen 2011
Narcissus tazetta ssp. *chinensis*	*A. tumefaciens*	Phytoene synthase (*psy*)	Intended to alter flower color (plants have not yet reached maturity.)	Lu *et al*. 2007
Ornithogalum dubium	Particle bombardment	OrMV replicase (*nib*) or coat-protein (CP) gene	Resistance to *Ornithogalum mosaic virus*	Cohen *et al*. 2005
Ornithogalum thyrsoides X *O. dubium*	*A. tumefaciens*	OrMV replicase (*nib*) or coat-protein (CP) gene	Resistance to *Ornithogalum mosaic virus*	Van Emmenes *et al*. 2008
Ornithogalum dubium	Particle bombardment	Tachyplesin 1 (TPN1)	Resistance to bacterial soft rot (*Erwinia*)	Cohen *et al*. 2011
Tricyrtis hirta	*A. tumefaciens*	Class B MADS-box genes from *Agapanthus*	Altered flower organs, including larger carpels and altered pigmentation	Nakano *et al*. 2007
Zantedeschia elliottiana	*A. tumefaciens*	Ferredoxin-like protein (*pflp*)	Resistance to *Erwinia* soft rot	Yip *et al*. 2007

formed with a combination of genes coding for subgroup II and the replicase gene. Some of the plants transformed with the replicase gene were found to be resistant to CMV subgroup I. In addition, some of the plants transformed with the gene for CMV subgroup II coat protein were resistant to the subgroup II virus (Kamo *et al*. 2010). Using an alternative approach, Kamo *et al*. (2012) attempted to introduce CMV resistance in Gladiolus by transforming the plants with genes coding for single-chain variable fragment antibodies (scFv) to CMV subgroup I or II. Although several transgenic plants were found to be more resistant than the non-transformed control, the frequency of the virus-resistant plants and the level of resistance was lower than the one reported earlier (Kamo *et al*. 2010) using the CMV coat protein and the CMV replicase genes.

Lipsky *et al*. (2002) reported on particle bombardment-mediated transformation with a defective replicase gene isolated from *Cucumber mosaic virus* (CMV), in an attempt to introduce virus resistance into *L. longiflorum* cv. 'Snow Queen'. The presence of the introduced gene was confirmed in this study. However, the transgenic plants were lost before a challenge with viral infection with the virus was ever attempted (pers. obs). There have also been attempts to introduce virus resistance into *Ornithogalum*. Particle acceleration (Cohen *et al*. 2005) and *Agrobacterium*-mediated transformation (van Emmenes *et al*. 2008) were used to introduce the coat protein (CP) gene or the replicase gene (*nib*) of the ornithogalum mosaic virus (OrMV) into *Ornithogalum*. The insertions of these transgenes were verified, but resistance following viral challenge has not yet been confirmed.

A different approach was taken by Wang *et al*. (2005). They transformed *Lilium longiflorum* with pokeweed antiviral protein (PAP), which is known to inhibit human viral infection. The gene was inserted and expressed in the leaves of the transgenic plants, yet there have been no reports on any subsequent resistance to viral infection.

There is great interest in using transformation methods to breed resistance to fungal or bacterial diseases into species and cultivars in which such resistance is not known to exist. Popowich *et al*. (2007) transformed *Hyacinthus orientalis* with the *thaumatin II* gene, in an attempt to confer resistance to *Fusarium culmorum* and *Botrytis cinerea*. They reported that their transgenic bulbs expressed substantial amounts of *thaumatin II* protein. Some of their transgenic lines were significantly more tolerant to *Botrytis* than

non-transformed control plants, although there was considerable variability in the levels of tolerance observed. One transgenic line was found to be more resistant, than the untransformed control, to *B. cinerea*; whereas another transgenic line was more resistant to *F. culmorum.*

Yip *et al*. (2007) introduced resistance to the bacterial soft rot induced by *Erwinia carotovora* (*Pectobacterium carotovorum*) into *Zantedeschia elliottiana* using *Agrobacterium*-mediated transformation with a ferredoxin-like gene (*pflp*) from sweet pepper. The gene was expressed in the transgenic plants, causing a hypersensitivity-like response in response to bacterial infection. Consequently, transgenic plants were more resistant to the bacterial soft rot. Similarly, Cohen *et al*. (2011) transformed *Ornithogalum dubium* with a gene coding for a small anti-microbial peptide, tachyplesin 1 (TPN1), originally isolated from the Japanese horseshoe crab, in order to introduce resistance to the bacterial soft rot. They reported that half of their transgenic plants remained healthy and flourished for months following the *Erwinia* challenge, despite the continued presence of the plant pathogen on and around the plants.

In an attempt to increase tolerance to salt, Yang *et al*. (2007) transformed *Lilium* with an ethylene response factor (ERF)–jasmonate an ethylene-responsive factor 3 protein (JERF3), which is known to activate the expression of oxidative-stress responsive genes in many plants, enhancing tolerance to salt, drought and freezing. They reported that the gene was over-expressed in the transgenic plants and enhanced the plants' salt tolerance. Chen *et al*. (2008) reported on the transformation of *Cyclamen* with the manganese superoxide dismutase (MnSOD) gene, in order to protect the plants from oxidative stress and, consequently, confer resistance to high-temperature stress. The overall physiological performance of the transgenic cyclamen plants subjected to high temperatures was better that that of the non-transgenic control plants.

3.2. Transformation for phenotypic alteration

Flower color and plant form are among the most important consumer traits in ornamental plants, including flowerbulbs. Transformation techniques offer the opportunity to add novel forms and new flower colors, beyond the range made possible by conventional breeding (reviewed by Boase and Davies 2006; Chandler and Tanaka 2007). Kiyokawa *et al*. (1996) introduced Ri *rol* genes from *A. rhizogenes* into *Begonia tuberhybrida* plants. The transgenic plants exhibited various degrees of dwarf growth habit, dark green leaves and wrinkled leaves and petals. The flowering time of these plants was also altered.

Similarly, Mercuri *et al*. (2003) transformed *Lilium longiflorum* cv. 'Snow Queen' with the *rol A*, *B* and *C* genes. The transformed plants exhibited various altered phenotypes, including a bushy growth habit, a dwarf appearance with compact internodes, fewer flowers per stem, a reduction in pollen fertility and over-developed root systems, in addition to shorter leaves and smaller florets.

Using the methods described by Cohen *et al*. (2004), we transformed the same *Lilium* cultivar with the target gene *rol B* driven by the anther-specific promoter *Lat*52, isolated from tomato in an attempt to generate pollen-less flowers (Cohen 2011). Although the transformation was successful and the presence of the target gene was confirmed in many of the resulting transgenic plants, only a few of the transgenic clones actually developed pollen-less flowers. Most of the transgenic clones were indistinguishable from the non-transgenic control plants. In addition, the expression, when present, seemed to vary as judged by the observed variation in phenotype. Some clones developed pollen-less flowers with empty anthers in one season (or flowering flush) and flowers with various degrees of pollen sterility in another. Pollen-less flowers were always much smaller than normal flowers, similar to the results reported by Mercuri *et al*. (2003).

Nakano *et al*. (2007) used class B MADS-box genes isolated from *Agapanthus praecox* to transform several monocot and dicot plants including *Tricyrtis hirta*. As expected, the transgene was able to cause morphological alteration in whorl 4 of the flower tepals, making them longer than those of the non-transgenic control. Changes in color and the spotting pattern were also evident, and the female reproductive organs failed to develop.

More recently, Li *et al*. (2008) reported on a transformation with *Zm401*, a pollen-specific gene from maize, reported to cause aberrant anther development and male sterility in transformed tobacco (Ma *et al*. 2005), in another attempt to develop pollen-less lilies. In this study, transformation was confirmed, but there was no evidence of the transgenic plants reaching flowering, the stage at which the trait should have been noticeable.

Mizukami *et al*. (2004) reported the creation of cyclamen plants with pale yellow flowers through transformation with the chalcone reductase gene. The yellow color was observed in the flower bud and during the early stages of flower opening.

In order to enhance the color of the foliage of *Caladium bicolor*, Li *et al*. (2005) transformed caladium explants with a maize anthocyanin regulatory gene (*Lc*). The expression of the transgene was correlated with enhanced pigmentation of the leaves, roots and stems of the otherwise green plants. Ku and Tsay (2009) attempted to introduce three genes involved in flavonoid biosynthesis (*DFR*, *F3H*, *CHI*) into *L. formosanum* seedlings that developed after the pollination of flowers with pollen mixed with an *Agrobacterium* culture. Few transgenic seedlings actually developed and the presence of myricetin-like flavonoids was detected in the bulbs and roots using thin layer chromatography (TLC), but could not be confirmed by HPLC.

Another attempt to alter flower color was carried out in *Narcissus tazetta*. Following an experiment using an antisense copy of the gene encoding phytoene synthase (PSY), a key regulatory enzyme in carotene biosynthesis, Lu *et al*. (2007) reported that the *psy* transcript was down-regulated to very low levels in the transgenic plants. However, it will not be possible to evaluate the effect of exogenous antisense-*psy* on the flower color of the transformants until the plants complete their 4-5 year juvenile phase and reach maturity.

More recently, Azadi *et al*. (2010b) transformed *Lilium* x *formolongi* with a cassette of seven genes of the ketocarotenoid biosynthetic pathway as the first step in the introduction of novel yellow-orange-red tepal colors into the white-colored lily. Large quantities of ketocarotenoids, with obvious orange color, were synthesized in both transgenic calli and leaves but the photosynthetic efficiency of the transgenic plantlets was significantly lowered. Following several sub-cultures, green plantlets developed with somewhat increased chlorophyll content and 31.4% elevation in total carotenoid content. The transgenic plantlets did not reach maturity, so the expression of the carotenoids in the tepals, and hence, novel flower colors, still remain to be seen.

Boase *et al*. (2010) were able to alter the color of *Cyclamen persicum* flowers by modifying the anthocyanin pathway, through antisense suppression of flavonoid 3',5'-hydroxylase, a key enzyme in anthocyanin biosynthesis. The overall concentration of the pigments was reduced and a shift from the delphinidin-derived pigments (malvidin or petunidin) towards peonidin- and cyanidin-based anthocyanin was observed.

4. The appropriate promoters

Appropriate promoters are another crucial component of a transformation system, as they assure the efficient expression of the genes involved in the system. Generally, the expression of both selectable genes and reporter genes is driven by constitutive promoters. The promoters that control the target genes may vary according to the time or developmental stage at which we would like them to be

expressed, or the tissue in which we wish them to be expressed. For example, genes that control resistance to insect pests and disease, including viral diseases, need to be expressed either constitutively or in response to exposure to the pest or pathogen. In contrast, genes involved in flower color have to be activated only in the petals; those which control fragrance need to be expressed in flowers only during anthesis and genes involved in pollen development must be expressed in the anthers during pollen development. Promoters are often species- or even cultivar-specific and are usually selected based on their ability to drive efficient transient expression of the reporter gene in the target tissue. In general, promoters isolated from monocots show higher activity in monocot species than in dicots and, in many cases, the presence of an intron in the 5′-untranslated region of the reporter gene enhances transient gene expression (reviewed by Wilmink *et al*. 1995b). However, Wilmink *et al*. (1995b) reported that in tulips, lilies and leek there was no significant difference between the activity levels of several monocot promoters and that of the cauliflower mosaic virus (CaMV) 35S. Furthermore, the presence of an alcohol dehydrogenase (*adh1*) intron in these liliaceous species inhibits GUS expression. Tsuchiya *et al*. (1996) found that the constitutive promoters, cauliflower mosaic virus (CaMV) 35S, maize alcohol dehydrogenase (*adh1*), rice actin (*Act1*) and maize ubiquitin (*ubi1*), differed in their ability to drive transient GUS expression in three different *Lilium* species.

The ability of seven different promoters to drive transient GUS expression in *Gladiolus* has also been examined (Kamo and Blowers 1999; Kamo *et al*. 2000a). The relative level of *gusA* expression in leaves of transformed plantlets grown *in vitro* was similar in the plants containing either CaMV 35S or *rolD* promoters and that this expression was higher than in plants that had mannopine synthase (*mas2*) or translation elongation factor1 subunit a (EF-1α) promoters (Kamo and Blowers 1999). The relative promoter activity was found to be tissue- and age-dependent (e.g., *rolD* delivered high levels of GUS expression in older leaves in which EF-1α and *mas2*-driven expression was rarely observed). All three promoters were associated with similar expression of GUS in younger leaves. The plants in which the transgene was under the control of EF-1α and *rolD* showed strong GUS expression in the root tips; whereas the plants in which the *mas2* promoter was used expressed GUS throughout the entire length of the root. Kamo (2003) reported that GUS expression in transgenic *Gladiolus* plants remained virtually unchanged following three seasons of dormancy and growing cycles and that there was little variation in GUS expression driven by the 35S, mannopine synthase (*mas2*), *Arabidopsis* ubiquitin (*UBQ3*) or *rolD* promoters. In all three cases, the level of expression in the roots was higher than that observed in the shoots. Expression patterns sometimes differ between *in vitro* and greenhouse-grown plants. Greenhouse-grown plants containing the *rolD* promoter expressed 4- to 11-fold greater amounts of GUS in their shoots and roots, respectively, as compared to plants grown *in vitro*. There is also an obvious discrepancy in the results concerning transient and stable expression. Kamo (2003) reported that the levels of transient GUS expression observed in *Gladiolus* were higher when the reporter gene was under the control of the *mas2* promoter than when the CaMV 35S or *rolD* promoters were used. In contrast, in stable transformants, the CaMV 35S delivered 7- to 28-fold higher levels of GUS expression than *mas2*. It has been suggested that the level of transient GUS expression may not reflect the level of gene expression in transgenic plants. In an attempt to direct higher levels of gene expression in *Gladiolus*, Kamo *et al*. (2009) compared GUS expression driven by two ubiquitin promoters (GUBQ2 and GUBU4) that were isolated from *Gladiolus*. Levels of GUS expression were relatively low in leaves of plants transformed with either promoters and higher in the roots and callus. GUBQ4 was found to be more efficient than GUBQ2 and both were less efficient than CaMV 35S. Yet, transgenic *Gladiolus* with the moderately expressing GUBQ2 and GUBQ4 were phenotypically normal compared to the slow-growing plants containing the 35S promoter. We can generalize that for any transformation attempt with any species or even with any specific clone, the most appropriate and efficient promoters will need to be identified empirically.

Pollen- or anther-specific promoters, such as the tomato *Lat*52, have been used in studies in lily (Van der Leede-Plegt *et al*. 1992; Nishihara *et al*. 1993; Miyoshi *et al*. 1995; SS Kim *et al*. 2007), including an attempt to produce pollen-less lily flowers (Cohen 2011). Van der Leede-Plegt *et al*. (1992) found the *Lat52* promoter to be silent in *L. longiflorum* cv. 'Gelria' pollen. They later used another promoter, *TR2'*, to drive GUS expression in the pollen grains (Van der Leede-Plegt *et al*. 1997). However, in another study (Nishihara *et al*. 1993), *Lat52* was found to be active in driving GUS expression in lily pollen. Tabata *et al*. (1993) found that the CaMV 35S promoter was able to drive GUS expression in callus-derived protoplasts and anthers, but not in microsporocytes. In contrast, meiotic promoters *mei2*Pro (Delta12 and 12-s) isolated from *Schizosaccharomyces pombe* directed GUS expression in protoplasts isolated from microsporocytes, but not in protoplasts isolated from somatic cells.

5. The active target tissue

The transformation and regeneration competence of target tissue is another crucial component of any genetic transformation. Although, in theory, every cell contains all the information necessary to generate an entire plant, in practice, cells in some organs respond better than others. For an efficient transformation system, the cells in the recipient tissue have to be able to accept foreign genes, integrate them into their genome and express them, while maintaining their capacity to regenerate into whole plants.

Since, in many flower-bulbs, plants can be regenerated from practically every tissue, the choice of active target tissue becomes a matter of calibration and personal preference. Various target tissues have been used to study of the expression of reporter genes and the calibration of gene-delivery methods, including stem segments (Langeveld *et al*. 1995), leaves (Wilmink *et al*. 1996; van Emmenes *et al*. 2008), bulbscales (Tsuchiya *et al*. 1996; Benedito *et al*. 2005; Thao *et al*. 2008), corms (Kamo *et al*. 1995a), immature embryos (Tsuchiya *et al*. 1996), filaments and styles (Hoshi *et al*. 2004; Krens *et al*. 2009) and pollen (Van der Leede-Plegt *et al*. 1992; Nishihara *et al.* 1993; SS Kim *et al*. 2007). However, most of the transformation attempts in flower bulbs have involved morphogenic or embryogenic callus tissue originating from various plant tissues [e.g., from leaves (Suzuki and Nakano 2002a, 2002b, in *Muscari*), tepals (Adachi *et al*. 2005, in *Tricyrtis*), stem segments (Lin *et al*. 2000, in *Alstroemeria*), bulbscales (Watad *et al*. 1998, in *Lilium*), hypocotyls (Boase *et al*. 2002, in *Cyclamen*) and ovules (Aziz *et al*. 2003, in *Hemerocallis*)]. (The reader should refer to the specific publications cited here for species-specific regeneration methods.) Following exposure to selective media, these calli were regenerated into transgenic plants.

Culture conditions also have a great influence on the success or failure of any attempted transformation, regardless of the gene-delivery system used. Initial attempts to transform a particular plant tissue usually yield many cells with transient expression of the transgene(s). Only in a small fraction of these cells is the transgene actually integrated into the plant genome and stably expressed. With greater numbers of transiently expressing cells, more stable integration is expected.

Tsuchiya *et al*. (1996) performed an experiment using scales taken from fresh *Lilium* bulbs and placed on a solid tissue culture medium. They found that the duration of the pre-culture of the bulbscales prior to particle bombardment greatly affected the number of transient blue spots. Cohen *et al*. (2004) reported that a reduction in the level of competence was evident in older cell cultures that had been

maintained in liquid culture for more than a year, despite the fact that they had been sub-cultured onto fresh media every two weeks. However, transferring the old cultures to fresh media one week prior to bombardment with the gene constructs restored the cells' competence to the levels observed in young tissue. The number of transient blue spots was found to be lower in cultures less than a week old, as well as cultures of more than one week (Cohen 2011). Furthermore, liquid-grown cultures always had a level of transformation competence 50-70 times greater than that of solid-grown cell clusters. In their paper describing *Agrobacterium*-mediated transformation of *Narcissus tazetta*, Lu *et al.* (2007) pointed out that the age of the culture prior to the co-cultivation is crucial for successful transformation, as determined by the frequency of transient GUS expression. They also determined that the window of opportunity for optimal transformation is 6-8 days after the cells were first cultured. Thereafter, the percentage of the explants expressing GUS drops dramatically.

Other reports have also highlighted the importance of the handling of the plants to be used for *Agrobacterium*-mediated transformation. For example, Ogaki *et al*. (2008) increased transformation efficiency in lilies by preventing the expected drop in the pH of the co-cultivation medium, to insure better survival of the bacteria. Other reports have indicated that better results can be obtained by using an NH_4NO_3-free co-cultivation medium (Hoshi *et al*. 2004; Li *et al*. 2008) or removing the macro-elements from the medium altogether (Azadi *et al*. 2010a, 2010b). Hoshi *et al*. (2005) reported a 3-fold increase in the number of transient GUS-expressing spots when the concentration of the gellan gum in the co-cultivation medium was increased. Culture conditions may have to be further calibrated whenever transformation is attempted in a new species or cultivar.

DISCUSSION

The ultimate purpose of using genetic engineering in the breeding of agricultural crops is the incorporation of a limited number of valuable traits that are not available in the original plant genome or in closely related species into improved cultivars that would otherwise lack such traits. There are many traits that can be targeted for genetic modification and many of the more economically significant crops, including ornamentals, have been successfully transformed (Zuker *et al*. 1998; Chandler and Lu 2005; Hammond 2006; Chandler and Tanaka 2007; Teixeira da Silva and Tanaka 2008).

In reviewing the numerous reports on genetic transformation in flower-bulb species, it becomes clear that most of the publications in this area have dealt with the calibration or optimization of transformation systems using marker genes, such as GUS, or selectable markers, such as herbicide or antibiotic resistance, rather than actual attempts to insert new economically important traits. Among the clones that were actually engineered with valuable traits, none have been commercialized or even registered to date. One can think of several reasons why this is so. First, there is the importance of the choice of the primary (parent) cultivar. It would be obvious to choose a cultivar that has high commercial value, but most flower-bulb cultivars are short-lived in the marketplace and, by the time the research has ended, are either out of fashion or have been replaced by newer, better cultivars. There are few cultivars that are more persistent, particularly among those that occupy specific market niches. An example of such a cultivar is the Easter lily cv. 'Nellie White', which is sold as a flowering potted plant for the highly specialized Easter market (pers. info.). Second, the huge number of flower-bulb cultivars and hybrids in the marketplace is a deterring factor. Since almost all flower-bulb cultivars are clonally propagated, the wish to insert even a single important trait would necessitate the calibration of the entire production system for each of the leading cultivars. Furthermore, the fact that a gene is inserted into the genome does not necessarily mean that it will be stably expressed. There is a need for further field trials and selection of the proper clones from among the somaclonal variants of those clones that are commercially valuable.

Public concerns and regulatory issues

Since flowers and ornamental plants are used mainly for ornamental purposes, one might expect that there would be less public resistance to transgenic ornamentals than to genetically modified (GM) crops used for human food and animal feed. In fact, both producers and consumers, especially in Europe and North America, still have to be convinced not only that transgenic cultivars have an advantage over the existing ones, but also that they do not pose a threat to human health and safety or to the environment. Such conviction has to be based on concrete data and such information is scarce if at all available. No legal distinction is made between GM ornamentals and other GM crops. The applicant who intends to register and commercialize a transgenic cultivar must go through a lengthy and expensive regulatory process that conventional breeders do not have to follow. This process sometimes requires separate applications and field trials in each country in which the cultivar would be distributed. These issues were thoroughly covered by Chandler and Tanaka (2007).

An important consideration in this process has to do with the nature of the genetic construct, more specifically, the target gene, the selectable marker, the reporter gene and their possible environmental impact. The EU encourages those who wish to release GM crops to develop marker-free plants in which only foreign DNA essential to the desired modification is present (Krens *et al*. 2004). This could be achieved either by avoiding the use of a selectable marker or through the use of a co-transformation process in which the selectable marker is placed on a separate construct and the resulting transgenic plants are crossed to generate segregants that carry the target gene, but not the selectable marker. Alternatively, the unwanted markers could be introduced into the plant with specific recombination sites on both sides and then physically removed from the transgenic plants when they are no longer needed, by treating the plants with a recombinase enzyme. These ideas were applied in *Lilium* hybrids (Krens *et al*. 2009), in a study in which the excision of the marker gene was accomplished by inducing recombinase activity in the transgenic plants.

Patented technologies and freedom to operate

Most of the publications on genetic transformation of horticultural crops, including flower-bulb species, have involved methods and technologies developed, at least in part, by others. Some or all of these technologies may be protected by intellectual property rights. These may include the original parent cultivar, the gene-delivery technology, the transformation vectors and the regulatory elements within them, the target gene(s) and the selectable and reporter genes. Most rightful owners of such technologies are willing to let researchers use them for scientific purposes, but the resulting transgenic cultivars cannot be commercialized without written authorization from those who own the various elements. Without this authorization, these transformation attempts, even those that produce promising results, are nothing but feasibility studies. Securing the license to use these elements from third parties may prove to be more expensive than the expected returns from the resulting cultivar(s), particularly in floricultural crops in which cultivars usually have rather short life-spans in the marketplace. Prior to starting new projects aimed at introducing novel genes for crop improvement and subsequent commercialization, it is imperative to make the necessary arrangements to ensure either full freedom to operate or that royalty payments will be bearable and not prohibitive.

CONCLUDING REMARKS

Most researchers working on the transformation of clonally propagated crops, like most flower-bulb crops, emphasize the advantages of using transformation techniques to introduce well-characterized genes directly into elite cultivars, maintaining their good quality traits and adding desired new ones. This statement represents an over-simplification of the process or, at best, wishful thinking. Efficient transformation yields a population of clones with considerable variation, as a consequence of the transformation process. Transgene expression depends on many factors, including the insertion site, copy number, position of the gene and the interaction of the gene with other genes, as well as its interaction with the environment. Selecting desirable clones may prove to be a lengthy process not necessarily shorter than the one required for conventional breeding. Coupled with the obstacles imposed by regulatory issues, risk assessment, intellectual property rights and public acceptance of GM crops, the financial burden is beyond the means of a small breeding company. The average breeder would be, perhaps, better off using transgenic cultivars as parents in his conventional breeding program and selecting marker-free plants from among the segregating seedling population. This would allow the breeder to avoid many, although not all, of the hurdles associated with registering transgenic cultivars and may help to ease public concerns.

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