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Transgenic Strawberry: Current Status and Future Perspectives

Miguel A. Quesada • Carmen Martín-Pizarro • Juan A. García-Gago • Sara Posé • Nieves Santiago • Rafael Sesmero • Fernando Pliego-Alfaro • José A. Mercado*

Departamento de Biología Vegetal, Universidad de Málaga, 29071, Málaga, Spain

Corresponding author: * **mercado@uma.es**

ABSTRACT

Strawberry (*Fragaria* × *ananassa* Duch.) world production has increased notably in the last 25 years, mainly due to the development of new cultivars adapted to a wide range of environmental conditions. However, the octoploid nature of this species, its high heterozygosity and the lack of natural resistance to several diseases, hamper the improvement of strawberry by conventional breeding. The high ability of strawberry tissues to regenerate *in vitro* has allowed the application of transgenic technologies to a high number of commercial cultivars, and genetic engineering appears now as a powerful tool to overcome some of the problems not resolved by conventional breeding. Most transformation studies in strawberry have been devoted to improve fruit quality and pest resistance, two of the major challenges in strawberry production. It is remarkable that positive results have been achieved in the manipulation of both traits and, for example, fruits with an extended postharvest shelf life or plants with enhanced tolerances to several fungal pathogens have been obtained. Strawberry is propagated vegetatively by runners instead of seeds. In spite of this handicap, the technology to eliminate marker genes has been tested successfully in this species. The application of these protocols opens the possibility to develop new transgenic plants free of unnecessary genes, which would facilitate the commercialization of transgenic strawberry. However, as it happens with other fruits, the negative public acceptance of genetically modified food may constrain the future development of transgenic strawberry. In this review, we outline the major trends in genetic transformation of strawberry, focusing on those recent advances that could be useful in its genetic improvement.

Keywords: *Fragaria*, *in vitro* tissue culture, genetic transformation, plant biotechnology

Abbreviations: 2,4-D, 2,4-dichlorophenoxiacetic acid; **5-FC**, 5-fluorocytosine; **BA**, N^6-bencyladenine; **CaMV35S**, cauliflower mosaic virus promoter; **CHS**, chalcone synthase gene; ***codA***, cytosine deaminase gene; **DMMF**, 2,5-dimethyl-4-methoxy-3(2H)-furanone; **GFP**, green fluorescent protein; **GUS**, β-glucuronidase reporter gene; **IBA**, indole butyric acid; **LEA**, late embryogenesis abundant proteins; **MS**, Murashige and Skoog medium; ***nptII***, neomycin phosphotransferasa gene; **TDZ**, thidiazuron; **TIB**, immersion bioreactor system

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INTRODUCTION

Cultivated strawberry (*Fragaria* × *ananassa*, Duch.) is one of the most valuable and worldwide cultivated small fruits. Its delicious sensorial attributes, attractive appearance and even its seasonal availability, make this fruit very attractive for consumers. Furthermore, strawberry fruit is rich in bioactive compounds with potent antioxidant power, which can contribute to diminish the risk of cancer and heart diseases (Hannum 2004). Consumer demand for strawberries increases every year, as well as world production. In the last 25 years, strawberry production has increased in linear progression, reaching over 3.5 millions tons in 2005 (FAOSTAT 2007). Besides its economical value, strawberry has been important as a model for physiological studies on non-climacteric fruit growth and development (Manning 1994; Perkins-Veazie 1995).

The genus *Fragaria* belongs to the Rosaceae family and comprises at least 23 species with a varying ploidy level (Folta and Davis 2006). The modern cultivated strawberry (*Fragaria* × *ananassa*, Duch.) is an octoploid hybrid (2n = 8x = 56) of recent origin. This species arose in Europe in the 18th century as a chance cross between two octoploid American native species, *F. virginiana* and *F. chiloensis*. This hybrid combined the large fruit size of *F. chiloensis* with the hermaphroditic character of *F. virginiana*. Intraspecific crosses of *F.* × *ananassa* have been extensively used to get modern cultivars with improved traits. However, the germplasm base is relatively narrow, and the majority of genes from modern cultivars came from few nuclear and cytoplasmic sources (Sjulin and Dale 1987; Dale and Sjulin 1990). Only two wild species, *F. vesca*, a diploid species with fragrant and small fruits, and *F. moschata*, a musky-flavored hexaploid, are also commercially grown on a

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reduced scale.

The continuous expansion of this crop is threatened by production practices currently used, that some considered as unsustainable (Pritts 2002). This crop has a high water requirement, use a large amount of plastic and pesticides and fumigation of the soil previous to the culture is required to avoid important loss of production. Regarding this last aspect, some authors estimate a 35-50% yield loss in the complete absence of soil fumigation (Sjulin 2003). The phase-out of methyl bromide, the most used fumigant in strawberry, under the Montreal Protocol in 2005 is therefore of great concern for strawberry producers, and great efforts are being devoted to find alternative fumigants (Sjulin 2003; Porter *et al*. 2006). The inconsistency of the fruit in the marketplace, the use of poor tasting cultivars, and the unaffordability of the product to poorer people have also been pointed out as main problems facing the strawberry industry. Dynamic strawberry breeding programs, both public and private, have been implemented in several countries to meet the problems above mentioned. As an example, Faedi *et al*. (2002) reported the release of 463 new varieties in the last 20 years. Among them, the most successful program has been developed by the University of California, which has released very popular varieties accounting for more than 50% of the world's strawberry production (Faedi *et al*. 2002). All breeding programs have similar objectives, i.e. fruit quality (size, flavour, firmness and color), yield and pest and disease resistance. However, the development of improved cultivars through classical breeding is hampered by the octoploid nature of this species, its high heterozygosity and its narrow germplasm base. The application of transgenic technologies to the improvement of the strawberry genome could provide a means to solve some of the limitations found with classical breeding. This is also facilitated by the amenability of strawberry tissues to its *in vitro* manipulation. Even more, as strawberry is propagated vegetatively rather than by seeds, genes can be integrated directly into elite cultivars, without the need of further breeding. First reports on strawberry transformation were published on 1990. Since then, the transformation methodology has been applied to numerous cultivars, and several genes controlling crucial traits, like fungal resistance or fruit firmness, have been introduced successfully. Current state of strawberry biotechnology and genomics has been recently reviewed (Graham 2005; Folta and Davis 2006; Debnath and Teixeira da Silva 2007; Mercado *et al*. 2007a). In this paper, we outline the major advances produced during the last years in the genetic transformation technology of strawberry, as well as in the application of this methodology to the genetic improvement of this crop.

STRAWBERRY TISSUE CULTURE

The development of an efficient system for *in vitro* shoot regeneration is an essential prerequisite to implement a successful transformation protocol. In this sense, strawberry can be considered as an amenable species to manipulate *in vitro*. Adventitious shoot regeneration has been obtained in many strawberry cultivars, using a broad range of explants, e.g. leaf disks (Jones *et al*. 1988; Nehra *et al*. 1989; Sorvari *et al*. 1993; Barceló *et al*. 1998), petioles (Jones *et al*. 1988; James *et al*. 1990; Rugini and Orlando 1992; Graham *et al*. 1995), stipules (Rugini and Orlando 1992), stem tissue (Mathews *et al*. 1995), runners (Liu and Sanford 1988), mesophyll protoplasts (Nyman and Wallin 1988), cotyledons (Miller and Chandler 1990), roots (Rugini and Orlando 1992), anthers (Owen and Miller 1996), ovaries (Passey *et al*. 2003), immature embryos (Wang *et al*. 1984), and sepals (Debnath 2005). Most of these regeneration protocols use Murashige and Skoog (1962) (MS) medium supplemented with 6-benzylaminopurine (BA) and indole-3-butyric acid (IBA). In general, shoot regeneration rates are high, although very genotype dependent. Passey *et al*. (2003) evaluated the parameters for optimal regeneration of seven commercial cultivars, as a first step through its genetic transformation. Four out of seven cultivars showed high regeneration rates, independently of the explant used, although in general, leaf disk was the explant with the best regeneration ability. By contrast, cvs. 'Elsanta' and 'Eros' showed a restricted ability to regenerate shoots, with regeneration rates between 2-8%. Interestingly, these authors found indications of a genetic linkage for *in vitro* recalcitrant behaviour when comparing the parentage among the different varieties with their regeneration rates. The variation in the regeneration capacity among different cultivars has also been reported in other papers (Jones *et al*. 1988; Nehra *et al*. 1989, 1990b), indicating a strong genetic component determining the success of adventitious regeneration. In an attempt to increase the regeneration efficiency of cv. 'Elsanta', one of the most important cultivars in Northern Europe, Passey *et al*. (2003) found a significant improve of the regeneration rate when using pre-pollinated ovaries as explant, achieving a 12% of regeneration. Similarly to *F.* × *ananassa*, leaf disk has been proved to be the most successful explant in the regeneration of the wild strawberry *F. vesca* (El Mansouri *et al*. 1996; Alsheikh *et al*. 2002).

In recent years, there has been an increased interest on the use of thidiazuron (TDZ) instead of BA on strawberry regeneration. TDZ has been shown to be very effective on shoot regeneration of woody species (Huetteman and Preece 1993), and its effect is not only due to its cytokinin-like activity but also related to its role as modulator of endogenous auxin levels. Landi and Mezzetti (2006) evaluated the effect of TDZ on shoot regeneration from leaf tissue of several strawberry cultivars. When using TDZ alone at 4.54 μM, most entries showed good regeneration, although the highest rates were achieved when TDZ was combined with IBA. Similarly, the best medium for 'Toyonoka' strawberry regeneration includes TDZ and IBA (Qin *et al*. 2005a). A MS medium supplemented with TDZ and IBA has been also used by Hanhineva *et al*. (2005) to optimize a protocol for shoot regeneration of five strawberry cultivars in a temporary immersion bioreactor system (TIB). According to these authors, TIB system allows shoot regeneration as efficiently as the conventional semi-solid medium, and reduces notably the handling of material and therefore the labor input. This regeneration protocol has been adapted to get successfully transgenic plants (Hanhineva and Kärenlampi 2007). Even though TDZ can improve regeneration, its use should be avoided in the proliferation phase because of an inhibitory effect of this plant growth regulator on shoot elongation (Debnath 2005, 2006).

Apart from genotype and growth regulators balance, other factors can influence strawberry regeneration. Thus, Landi and Mezzetti (2006) reported a stimulatory effect of continuous darkness incubation of explants on shoot regeneration. However, this effect can be also genotype dependent since the contrary result was reported by Barceló *et al*. (1998) in the regeneration of cv. 'Chandler'. The addition of silver nitrate to the regeneration medium speed up the initiation of adventitious buds and enhanced shoot regeneration rates (Qin *et al*. 2005b). This chemical has a significant stimulatory effect on chlorophyll and soluble protein contents as well as antioxidant enzyme activities. The last one effect can be the most important, since Tian *et al*. (2003) have found that endogenous H_2O_2 and antioxidant activities correlate with the mophogenetic process in strawberry calli. Even more, the enhanced effect of color plastic films on regeneration of strawberry cv. 'Toyonoka' was related to an increment on antioxidant enzyme activities (Qin *et al*. 2005a).

An important issue that it is frequently omitted in regeneration studies of strawberry is the incidence of somaclonal variation. Genetic variations induced by *in vitro* tissue culture techniques have been perceived as an additional source of variability, particularly for improvement of asexually propagated plants. Recent works on strawberry demonstrate the potential use of somaclonal variation to improve several traits like resistance to fungal pathogen (Toyoda *et al*. 1991; Sowik *et al*. 2001), low temperature (Ru-

gienius and Stanys 2001) and salt stress (Dziadczyk *et al*. 2003). However, somaclonal variation is a potential nuisance when tissue culture is used for genetic engineering, because it can lead to phenotypes in transgenic lines not directly related to the transgenes inserted into the plant genome (Bhat and Srinivasan 2002). In the case of strawberry, commercial micropropagation in media with high concentrations of growth regulators to enhance axillary bud proliferation can induce a relative high proportion of somaclonal variation, being the chlorosis and dwarfism the most common variants observed (Sansavini *et al*. 1990). In adventitious shoots, Nehra *et al*. (1992) reported a reduction on plant vigor, petiole length and leaf size in plants regenerated from leaf callus. By contrast, the number of leaves and runnering ability were enhanced in regenerated plants. These effects disappeared in the runner progeny under field conditions. Dwarf variants were obtained in this report only when shoots were regenerated from callus cultured at high hormonal concentration, 20 µM each of BA and 2,4-D, and leaf shape and yellow leaf variants were also obtained in shoots regenerated from old calli with more than 16 weeks of culture (Nehra *et al*. 1992). Jones *et al*. (1988) also reported the occurrence of a high percentage of somaclonal variation in shoots regenerated from leaf or petiole calli. Similarly, the regeneration of strawberry plants from protoplasts induces a high frequency of chromosomal alterations and somaclonal variation (Nyman and Wallin 1992). At the molecular level, Brandizzi *et al*. (2001) performed an analysis of DNA content by flow cytometry on five strawberry cultivars propagated by runners or regenerated *in vitro* from callus. It is noteworthy that all callus derived plants showed an additional DNA peak besides the 2C present in runner plants. However, in four out of five cultivars, this peak disappeared after transfer to the greenhouse. In conclusion, it seems clear that an elevated hormonal concentration in the medium, an extended regeneration phase from callus, and the use of protoplasts should be avoided to improve strawberry through genetic engineering.

GENETIC TRANSFORMATION TECHNOLOGY

Genetic transformation of cultivated strawberry was first reported in 1990 by two independent groups (James *et al*. 1990; Nehra *et al*. 1990a), using both *Agrobacterium tumefaciens* infection. A report on wild strawberry, *F. vesca*, transformation was published six years later (El Mansouri *et al*. 1996). Since then, protocols for genetic transformation have been optimized for many strawberry genotypes, including most popular cultivars such as 'Chandler', 'Camarosa', 'Elsanta', 'Pajaro'. *Agrobacterium tumefaciens*-mediated transformation is the method most commonly employed, although there are few reports on transgenic strawberry obtained by using biolistic (Wang *et al*. 2004) and protoplast electroporation (Nyman and Wallin 1992). A method combining *Agrobacterium* infection and biolistic bombardment has also been developed (Cordero de Mesa *et al*. 2000). Independently of the method used, the transformation efficiencies are, in general, low, ranging from 1 to 10%, and extremely genotype dependent. **Fig. 1** shows different steps of strawberry transformation mediated by *Agrobacterium* infection.

A number of factors affect the success of *Agrobacterium* transformation, e.g. type of explant, preculture of explants prior to inoculation, *Agrobacterium* virulence and selection strategy. Comprehensive analysis of these factors have been recently reviewed (Mercado *et al*. 2005; Folta and Dhingra 2006; Mercado *et al*. 2007a). Selection phase is possible one of the most critical steps in the success of *Agrobacterium* transformation. Most binary vectors used to transform strawberry are derived from pBIN19 (Bevan 1984), carrying the *nptII* gene for kanamycin selection. Strawberry tissues are extremely sensitive to this antibiotic and concentrations as low as 10 mg/l impair shoot regeneration (Barceló *et al*. 1998; Alsheikh *et al*. 2002; Gruchala *et al*. 2004). Then, true transgenic plants can be recovered

Fig. 1 Different steps of *Agrobacterium* mediated transformation in strawberry, cv. 'Chandler'. (**A**) Putative transgenic shoots regenerated in the medium described by Barceló *et al*. (1998), in the presence of 25 mg/l kanamycin. (**B**) Transgenic shoots micropropagated, in a medium supplemented with 25 mg/l kanamycin. (**C**) Transgenic plants growing in the screenhouse for phonotypical analysis.

by cultivating the explants in a selection medium with a moderate to low kanamycin concentration. However, some cultivars show a high tolerance to kanamycin and require a different selection strategy. Mathews *et al*. (1995, 1998) found that an elevated percentage of plants, cv. 'Totem', recovered in a medium with 25 mg/l kanamycin were chimeras, plants containing transgenic and non-transgenic sectors. To avoid this problem and obtain pure transgenics, these authors developed a selection procedure based on recurrent adventitious regeneration in media with increasing kanamycin levels. Thus, *in vitro* leaves from the primary transgenics, obtained on a selection medium with 25 mg/l kanamycin, were subjected to three regeneration cycles, stepwise increasing the kanamycin level from 40 to 80 mg/l. This procedure has been protected under the US patent no. 5,750,870. Besides genotype, explant type can also influence the percentage of chimaerism. Although few reports describe chimaerism on adventitious shoots regenerated from leaf explants, this is not the case when using stipules, and the risk of recover chimaeric plants seems to be high with this material (Monticelli *et al*. 2002; Chalavi *et al*. 2003). It can be concluded that although recurrent regeneration can avoid the chimaerism, this procedure should be avoided as the risk of somaclonal variations is notably increased.

Apart from its economical importance, strawberry has been proposed as a model species for translational genomics for the *Rosaceae* (Folta and Dhingra 2006), a family which includes many valuable fruit and ornamental crops as peach, apple or roses. Translational genomics is defined as the application of molecular approaches devised from model systems such as *Arabidopsis thaliana* to species of agronomical interest. The main advantages of strawberry against other *Rosaceae* members are its herbaceous perennial character, small size, rapid growth, high ability to manipulate *in vitro*, and, in the case of *F. vesca*, its small genome size (200 Mb, slightly higher than the 164 Mb genome of *A. thaliana*). However, although strawberry is easily transformed, high efficient transformation methods are needed to accomplish this goal. Along this line, recent reports have developed new procedures that increase the speed and efficiency of genetic transformation in both a diploid and octoploid strawberries. Oosumi *et al*. (2006) optimized the conditions for genetic transformation of *F. vesca*, accession 'Alpine' achieving a 42% transformation rate. The main advances of this protocol were the use of stringent selection on MS medium containing 4 mg/l hygromycin and the use

of GFP for transgenic cell screening. In this protocol, explants obtained from unfolded leaves were cut into 4 to 6 secondary explants before transfer to selective medium, about six days after inoculation with *Agrobacterium* strain GV3101. Later, explants were screened by GFP expression and GFP positive calli with apparent independent cellular origin were isolated to get tertiary explants. Several *F. vesca* accessions were tested obtaining a high variability in transformation efficiencies. The most efficient, and therefore the best candidate for functional genomics, was the genotype 'Hawaii-4' (accession PI 551572) which showed a 100% transformation rate considering the primary explants. More than 500 independent transgenic lines were produced using this protocol. Based on the genome size of diploid strawberry, at least 255,000 independent T-DNA lines should be required to mutate any single gene in *F. vesca* (Oosumi *et al*. 2006). In the case of octoploid strawberry, Folta *et al*. (2006) developed a high-throughput transgenic system based on the use of a new genetic line, LF9, selected for rapid regeneration from seedlings of strawberry cv. 'Festival'. In this system, petioles were infected by *Agrobacterium* and selected under low kanamycin concentration, 2.5 mg/l for 14 days followed by selection on 5 mg/l. The transformation frequency based on number of explants producing at least one shoot was approximately 75%. Although T-DNA mutagenesis is difficult to apply to cultivated strawberry due to its vegetatively propagation character, high efficient transformation systems similar to the one above described should be useful to transfer knowledge achieved in diploid strawberry to commercial cultivars, by rapidly testing gene function in the field. In this context, as LF9 cultivar has been obtained from seedlings of cv. 'Festival', the usefulness of this new cultivar as model system would require a complete phenotypic analysis in field conditions, because important traits like fruit quality and yield could be significantly different from the original 'Festival' plant.

A significant recent advance in the strawberry transformation technology has been the successful application of a system to remove marker genes (Schaart *et al*. 2004). Although there are not any evidences of adverse effects of marker genes on human health and the environment (Ramessar *et al*. 2007), public concerns about antibiotic resistance genes, especially in Europe, are demanding that transgenic plants to be commercialized are devoid of unnecessary genes, marker genes and/or backbone sequences. Along this line, a recent report of Abdal-Aziz *et al*. (2006) showed that more than 65% of transgenic strawberry lines transformed with pBIN19 derived vectors contained non-T-DNA backbone sequences. Among the different strategies developed to obtain marker-free plants (see Ebinuma *et al*. 2001), those based on co-integration of two T-DNA, the simplest system, can not be applied to vegetatively propagated species because of the need of sexual crossing to segregate marker gene and the gene of interest. Schaart *et al*. (2004) developed a transformation system to remove marker genes in transgenic strawberry based on the use of an inducible site-specific recombinase. These authors used a binary vector containing a plant-adapted recombinase (*R*) gene and a bifunctional selectable marker, combining positive selection, *nptII* gene, with negative selection, cytosine deaminase, *codA*, gene. Both genes were located adjacent and flanked by directly repeated recombination sites. After shoot regeneration on kanamycin, leaf explants from kanamycin resistant shoots were treated with dexamethasone to induce the recombinase gene and subsequently subjected to a second round of regeneration in the presence of 5-fluorocytosine (5-FC). The *codA* gene converts the non-toxic 5-FC to cytotoxic fluorouracil (Stougaard 1993), leading to the dead of cells still containing the marker genes. After negative selection, 30 putative marker-free transgenic plants could be recovered.

Independently of the cultivar and transformation protocol, the obtainment of stable transgenic lines to study the function of genes, specially those related to fruit ripening, is a laborious and time-consuming task. To test ripening related genes in a rapid way, Hoffmann *et al*. (2006) optimized the conditions for RNAi silencing by agroinfiltration of developing fruits attached to the plants, based in a previous protocol of Spolaore *et al*. (2001). They used a construct containing a sense and its corresponding antisense sequence of a chalcone syntahse gene (CHS) separated by an intron. After agroinfiltration, the silencing of the CHS gene was monitored by the absence of red coloration in ripened fruits. Optimal conditions for maximum silencing were three *Agrobacterium* infiltrations performed between 10 and 14 days post-pollination, when the fruits had almost gained their maximum size.

PRACTICAL APPLICATION OF GENETIC TRANSFORMATION TO IMPROVE SELECTED TRAITS

Since 1992, at least 24 papers dealing with genetic transformation of strawberry to manipulate important traits have been published in peer-reviewed journals. This information is summarized in **Table 1**. Most of these works have been devoted to improve fungal resistance and fruit quality, reflecting the main concerns of strawberry industry. As previously stated, these are general objectives of most breeding programs.

Biotic stress

Strawberry cultivation requires almost a complete soil sterilization to assure good fruit yield (Sjulin 2003). As a nearly universal practice, strawberry soil sterilization has been accomplished by preplant fumigation with methyl bromide, but due to its negative effect on the tropospheric ozone its use has been forbidden in developing countries under the Montreal Protocol. In spite of the efforts performed to find alternatives, nowadays, we lack environmentally friendly substitutes for methyl bromide, especially for nursery production (Porter *et al*. 2006). One of the alternatives to the use of chemicals to control strawberry diseases is the introduction of resistant varieties. However, although some level of variability on resistance have been found within the strawberry germplasm, for some diseases such as *Verticillium* wilt (Shaw *et al*. 1996) or anthracnose (Casado-Díaz *et al*. 2006) no totally resistant genotypes have been detected. For other pests, there are not sources of resistance on neither cultivated nor wild strawberry (Rosskopf 1999). Transgenic technology could help to solve this problem in the long term, and also would improve the safety and quality of this crop by reducing the use of pesticides.

Fungi are the most important strawberry pathogens in terms of yield loss. Major fungal diseases include red stele (*Phytophtora fragariae*), *Verticillium* wilt, leaf spot (*Mycospharella fragariae*), leaf scorch (*Diplocarpon earlianum*), powdery mildew (*Sphaeroteca macularis*), gray mold (*Botrytis cinerea*) and anthracnose (*Colletotrichum* spp.) (Maas 1998). In recent years, introduction of resistance transgenes against some of these diseases has proven very promising. Asao *et al*. (1997) first reported the introduction of an antifungal gene into strawberry. They overexpressed a rice chitinase gene in cv. 'Toyonoka'. Transgenic plants showed higher level of chitinase activity than control plants and a significant reduction in the disease symptoms caused by *Sphaeroteca humulis*. In the field, yield of transgenic plants was similar to controls, and interestingly, no significant environmental effects on the growth of other plants and microflora were detected (Asao *et al*. 2003). Chalavi *et al*. (2003) also introduced a chitinase gene to enhance resistance to *Verticillium dahliae*. After infection with this pathogen, the three independent transgenic lines obtained showed a low rate of crown infection and lower wilting symptoms than non-transformed plants. Gray mold, caused by *Botrytis cinerea*, is responsible for significant pre and post-harvest yield loss. This fungus attack different organs and it is easily spread by rain. Flowers are usually infected at blossom, and the long latency period until the appearance

Table 1 Summary of traits that have been manipulated by genetic transformation in strawberry.

Trait	Gene	Cultivar	Reference
Fungal resistance			
Verticillium dahliae	Chitinase from *L. chilense*	Joliette	Chalavi *et al*. 2003
Sphaeroteca humuli	Chitinase from rice	Toyonoka	Asao *et al*. 1997
Botrytis cinerea	Chitinase from *Phaseolus vulgaris*	Pajaro	Vellicce e*t al*. 2006
	Thaumatin II from *Thaumatococcus danielli*	Firework	Schestibratov and Dolgov 2005
Colletotrichum acutatum	Chitinase and glucanase from *Trichoderma harzianum*	Camarosa	Mercado *et al*. 2007b
Insect resistance			
Otiorynchus spp.	Cowpea protease inhibitor	Rapella	James *et al*. 1992
		Symphony	Graham *et al*. 1995
Virus resistance			
Mild yellow edge virus	Coat protein		Finstad and Martin 1995
Herbicide tolerance			
Glyphosate resistance	EPSP from *A. tumefaciens*	Camarosa	Morgan *et al*. 2002
Glufosinate	PAT	Selekta	du Plessis *et al*. 1997
Abiotic stress			
Salt tolerance	LEA3 from barley	Toyonaka	Wang *et a*l. 2004
Cold tolerance	Acidic dehydrin from wheat	Chambly	Houde *et al*. 2004
	Cold-induced transcription factor CBF1 from *Arabidopsis*	Honeoye	Owens *et al*. 2002
Fruit quality			
Reduced fruit softening	S-adenosylmethionine hydrolase from T3 bacteriophage	Totem	Mathews *et al*. 1995
	Strawberry glucanase *cel1* (AS)	Calypso	Woolley *et al*. 2001
	Strawberry glucanase *cel2* (AS)	Calypso	Palomer *et al*. 2006
	Strawberry pectate lyase (AS)	Chandler	Jiménez-Bermúdez *et al*. 2002
Increased sugar content	ADP-glucose pyrophosphorylase (AS)	Anther	Park *et al*. 2006
Increased fruit size	Auxin gene *iaaM* from *Pseudomonas syringae*	Breeding selection	Mezzetti *et al*. 2004
Fruit pigmentation	Strawberry chalcone synthase (AS)	Calypso	Lunkenbein *et al*. 2006a
Fruit aroma	Strawberry *O*-methyltransferase (AS)	Calypso	Lunkenbein *et al*. 2006b
Other studies			
Modified auxin metabolism	IAA-glucose synthase from maize	Kaster	Wawrzynczak *et al*. 2005
Promoter for fruit expression	Receptacle promoter from petunia	Polka and Gariguette	Schaart *et al*. 2002
Promoter for phloem expression	Sucrose-H^+ symporter gene promoter from *Arabidopsis*	*F. vesca*	Zhao *et al*. 2004

AS: antisense orientation

of the symptoms makes difficult the control of this pathogen with fungicides. Two strategies have been employed to get transgenic plants resistant to *Botrytis*. Schestibratov and Dolgov (2005) introduced the thaumatin II gene from *Thaumatococcus danielli* into strawberry cv. 'Firework'. This gene codifies a phatogenesis-related protein with antifungal activity, and it is also associated with sweet taste (Edens *et al*. 1982). In a leaf disk assay, transgenic plants showed enhanced resistance to *Botrytis*. In the other hand, Vellicce *et al*. (2006) obtained transgenic plants, cv. 'Pajaro', containing a chitinase from *Phaseolus vulgaris*, a glucanase or a thaumatin-like protein, both from *Nicotiana tabacum*. Only two lines containing the bean chitinase showed enhanced resistance to *Botrytis*. However, none showed resistance to anthracnose. This last disease, caused by several *Colletotrichum* species, is considered one of the most important on strawberry industry, causing lesions on fruits, crown, petioles and stolons (Casado-Díaz *et al*. 2006). Recently, we have found that the expression of a β-1,3-glucanase gene isolated from the antagonist soil fungus *Trichoderma harzianum* enhanced anthracnose resistance (Mercado *et al*. 2007b). Three out of eight transgenic lines analyzed showed lower disease symptoms in crowns, leaves and petioles. By contrast, the overexpression of the *Trichoderma* chitinase *chit42*, resulted in a significant improvement of *Colletotrichum* resistance in only one out of six lines analyzed (Mercado *et al*. 2007b).

Insect resistance in transgenic strawberry has been achieved through the introduction of antifeedant genes. James *et al*. (1992) and Graham *et al*. (1995) obtained transgenic plants expressing a cowpea protease inhibitor (*CpTi*). The product of this gene forms complexes with proteinases, inhibiting proteolitic activity and interfering with development of many Lepidopteran and Coleopteran larvae. In both cases, plants were tested for resistance to vine weevil larvae (*Otiorynchus* spp.) with contrasting results. James *et al*. (1992) carried out survival bioassays in six transgenic clones but they did not found any reduction in the survival rate of the insect when using transgenic plants. By contrast, Graham *et al*. (1997, 2002) found that transgenic plants were less damaged than controls when infected by weevil larvae under glasshouse and field conditions. According to Graham (2005), a lectin gene isolated from *Galanthus nivalis*, a different anti-insect protein, has also been introduced into strawberry, alone or in combination with *CpTi*, but transgenic plants failed to show resistance to weevil attack.

Weed control in strawberry nursery and fruit production in the absence of methyl bromide fumigation is problematic, since chemical alternatives have inadequate herbicidal components (Morgan *et al*. 2002). Herbicide tolerant plants have been obtained by du Plessis *et al*. (1997) and Morgan *et al*. (2002). In the first case, the phosphinotricin acetyl transferase gene was introduced into cv. 'Selekta' to get resistance against glufosinate. Field trials analysis of transgenic plants showed that four out of 22 herbicide resistant lines resembled the phenotypic characteristics of untransformed control plants under commercial conditions. In the other hand, Morgan *et al*. (2002) obtained transgenic Camarosa plants resistant to glyphosate, the active compound of Roundup, through the introduction of ESPS synthase gene from *A. tumefaciens*. Transgenic plants showed a broad range of tolerances, from complete tolerance to death, to commercial levels of glyphosate applied by spraying at the nursery. Southern analysis revealed that 93% of the selected transgenic lines contained a single gene insertion, and plasmid backbone sequences were detected in 13% of lines.

As far as we know, there has been only one attempt to obtain transgenic strawberry plants resistant to virus. Finstad and Martin (1995) described the introduction of the coat protein gene from strawberry mild yellow edge virus, although virus tolerance of transgenic plants was not reported.

Abiotic stress

To increase resistance to salt and drought stress, Wang *et al*. (2004) introduced a late embryogenesis abundant protein gene from barley, LEA3, into strawberry. LEA proteins have been found in a wide range of plant species in response to water deficit resulting from desiccation, osmotic and cold stress (Wang *et al*. 2003). Although its function is largely unknown, it has been suggested that LEA-type proteins act as a water-binding molecules, in ion sequestration and in macromolecule and membrane stabilization. When strawberry plants were cultured *in vitro* in a medium supplemented with 50 or 100 mM NaCl for 7 days, the transgenic lines overexpressing the LEA3 gene showed a significant reduction in the rate of wilting when compared with control plants. Additionally, after removing salt stress, the recovery of transgenic shoots was much better than controls. Houde *et al*. (2004) improved the freezing tolerance of strawberry plants by incorporation of a dehydrin gene, *wcor410*, isolated from wheat. Dehydrins are a different class of LEA proteins, hypothetically involved in the stabilization of plasma membrane during dehydration associated with freezing stress. They obtained three transgenic lines which expressed the protein at high levels. All lines showed a 5ºC reduction in the threshold temperature of freezing damage on leaves when plants were previously cold-acclimated, lowering from -12ºC to -17ºC. However, this improvement on freezing tolerance was not observed on non-acclimated plants, suggesting that WCOR410 protein needs to be activated by another factor induced during cold acclimation. When whole plants were subjected to freezing conditions, transgenic plants collapsed during the recovery phase, indicating that roots do not benefit from the overexpression of WCOR410, maybe as result of a low accumulation of the factor needed to activate the dehydrin protein. Owens *et al*. (2002) employed a different strategy to enhance cold tolerance in strawberry. They obtained two transgenic lines overexpressing the cold-induced transcription factor CBF1 from *Arabidopsis*. Leaf disks of transgenic plants showed an improvement on the freezing tolerance, however, no differences between wild type and transgenic lines was detected on fruit receptacle. The success on the cold resistance of berries could increase postharvest fruit shelf life, with a consequent high economic impact.

Fruit yield and quality

In spite of its polygenic character, fruit production can be increased through transformation with a single construct. Mezzetti *et al*. (2004) transformed diploid and octoploid strawberry with the coding region of the *iaaM* gene from *Pseudomonas syringae*, an auxin-producing gene, under the control of the regulatory region of the *DefH9* gene from snapdragon, which promotes expression in placenta/ovules. Transgenic plants of both species showed an increased number of inflorescences and also more flowers per inflorescence, increasing fruit yield by 180% in cultivated strawberry and by 140% in wild strawberry.

Taste, texture, aroma, color and nutritional composition are main attributes of strawberry fruit quality. In recent years, there have been several examples on how transgenic technology can help to improve these traits. One of the main problems of strawberry industry is the postharvest losses due to the rapid deterioration of the fruit. Strawberry soften rapidly, acquiring a melting texture in few days after ripening, limiting its shelf life. In general, there are two different strategies to control fruit softening, to delay ripening by inhibition of ethylene synthesis or to down-regulate enzymes involved in cell wall degradation, since it is generally accepted that fruit softening is mainly associated with cell wall disintegration (Brummell and Harpster 2001). Strawberry fruit has traditionally been considered as non-climacteric because its ripening is not associated with significant changes in ethylene production (Perkins-Veazie 1995). However, some evidences indicated that ethylene may play a role in strawberry ripening. In fact, the use of laser photoacustic spectroscopy to detect very low levels of ethylene indicates that this fruit could be classified as climacteric (Iannetta *et al*. 2006). Therefore, the reduction of ethylene biosynthesis could increase strawberry postharvest shelf life. Along this line, Mathews *et al*. (1995) described the transformation of strawberry with the S-adenosylmethionine hydrolase from T3 bacteriophage. This gene reduces the level of ethylene precursors. Although, plants were confirmed to be transgenics, the effect of ethylene reduction on fruit ripening was not reported.

Regarding the second strategy, Jiménez-Bermúdez *et al*. (2002) obtained transgenic plants containing an antisense sequence of a pectate lyase gene under the control of the constitutive promoter CaMV35S. Ripened fruits from transgenic lines were significantly firmer than control fruits and showed a reduced postharvest softening, extending their shelf life. A negative consequence of pectate lyase inhibition was the reduction on fruit yield in most of the lines, likely due to the role of this gene in pollination. Recently, we have observed that transformation with a sense construct of this gene reduces the mRNA pectate lyase level and enhanced fruit firmness, without affecting fruit yield (unpublished results). The inhibition of this gene in ripen fruits also improves quality of strawberry derived foods. Thus, Sesmero *et al*. (2007) found that jam prepared with transgenic fruits contained more berries, one of the main parameters of jam quality, and cooked berries were also firmer than control berries. Unlike these results, the inhibition of two strawberry cellulase genes, *cel1* and *cel2*, did not increase fruit firmness on cv. 'Calypso' (Woolley *et al*. 2001; Palomer *et al*. 2006).

To modulate the soluble sugar content of strawberry fruits, Park *et al*. (2006) generated transgenic plants, cv. 'Anther', containing an antisense cDNA of an ADP-glucose pyrophosphorylase small subunit. This gene catalyzes a key step in starch biosynthesis, and its down-regulation would reduce starch content. The antisense sequence was under the control of a strawberry ascorbate peroxidase promoter which showed strong expression in ripe fruit. Transgenic ripe fruits contained a lower amount of starch whereas the soluble sugars content was slightly increased when compared with non-transformed type fruits.

Color and aroma of ripen strawberry fruits have recently been studied by transgenic approaches. Lunkenbein *et al*. (2006a) obtained transgenic plants containing an antisense chalcone synthase gene to analyze the effect of down-regulation of this gene on pigment accumulation in ripen fruit. Reduction of the CHS function by antisense technology has mainly been used to change flower pigmentation. In strawberry fruit, a 95% reduction on the CHS expression was required to affect pigmentation. Unexpectedly, the inhibition of CHS not only modified the level of metabolites downstream the pathway but also the compounds upstream of the CHS branching point, indicating a key role of this gene in secondary metabolism regulation.

One of the most important components of strawberry aroma is 4-hydroxy-2,5-dimethyl-3(2H)-furanone. This compound is methylated during the ripening process to 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF) by a *O*-methyltransferase enzyme. Lunkenbein *et al*. (2006b) down-regulated this gene in transgenic strawberry, obtaining a near total depletion of DMMF in several lines, without affecting the levels of other volatiles. The inhibition of the *O*-methyltransferase gene also decreased significantly the level of the phenolic compound feruloyl-β-D-glucose.

Other transgenic studies

Transgenic technology has also been used to study different physiological processes in strawberry or to analyze the usefulness of heterologous promoters. Wawrzynczak *et al*. (2005) modified endogenous auxin metabolism in strawberry through the inclusion of a maize IAA-glucose synthase gene. Auxin analysis of transgenic leaves showed an

increased level of ester-conjugated IAA, and for several lines, a significant reduction on free IAA. Phenotypically, transgenic plants were dwarfish, and formed more roots *in vitro* than control plants.

Schaart *et al*. (2002) obtained plants expressing the GUS marker gene under the control of a receptacle promoter (*FBP7*) from petunia to test its utility as a fruit-specific promoter. Histochemical GUS activity was only detected in reproductive tissue. However, although GUS staining was not observed in roots, northern analysis showed a significant GUS mRNA level in this tissue. These authors concluded that this promoter could be useful to modify receptacle-specific traits, although the level of expression in fruit is relative lower in comparison to a constitutive promoter as CaMV35S, and low expression on roots is also expected to occur. Cordero de Mesa *et al*. (2004) evaluated the expression of the CaMV35S promoter in flowers and pollen of transgenic strawberry plants. Contrary to the general view, the 35S promoter was highly active in mature pollen, and even its expression was maintained in pollen stored for 5 weeks at room temperature. Finally, to enhance tolerance to phytoplasmas, prokaryotes restricted to sieve elements, Zhao *et al*. (2004) produced transgenic plants expressing the *Arabidopsis* sucrose-H^+ symporter gene (*AtSUC2*) promoter. As expected, histological GUS activity was restricted to phloem tissues of leaves, petioles and roots of transgenic plants, demonstrating its utility for achieving phytoplasm resistance.

RISK ASSESSMENT OF TRANSGENIC PLANTS

Before genetic modified plants can be commercially cultivated, a risk assessment to compliance with environmental and human health safety is required under national and international legislation. Among others aspects, risk evaluation should include the assessment of the rates of gene flow between GM crops and wild relatives, the potential for transferred material to cause an adverse effect in the new genetic background, and an assessment of substantial equivalent, i.e. determining whether the GM crop is as safe as its traditional counterpart in terms of agronomic, compositional, safety and nutritional equivalence (Cockburn 2002). Flavr Savr® tomato with delayed fruit ripening was the first transgenic crop approved for sale in 1994, but due to market issues this transgenic crop is not longer commercialized. Besides tomatoes, transgenic papaya and cantaloupe melons have completed the regulatory agency reviews for planting and food within the US (http://usbiotechreg.nbii.gov/database_pub.asp). However, no transgenic fruits are currently being commercialized (Nickson 2005). Thus, information about risk assessment of transgenic strawberry is really scarce. A crucial component for a proper risk assessment is defining the appropriate baseline for comparison and decision. For GM crops, the best reference point is the impact of plants developed by traditional breeding, in the context that existing foods have a long history of safe use (Cockburn 2002), and many putative impacts identified for GM crops are very similar to the impacts of new varieties derived from traditional breeding (Nap *et al*. 2003). The comparison with existing crops for safety evaluation should be an holistic approach, performed step wise and on a case-by-case basis, also taking into account all changes involved in the development of the new GM variety, i.e., the characteristics of the parent crop, the gene donor and transformation procedure, the newly expressed gene product, and the characteristics of the new crop, food or processed product (Cockburn 2002). Regarding environmental risk assessment of GM crops, Conner *et al*. (2003) pointed out several questions that should be addressed, i.e. the ability of the transgenic crop to invade agricultural and natural ecosystem, the transfer of transgene to related species, the contribution of GM crop to horizontal gene transfer, the risk of ecological secondary impact, the likelihood that GM crop lead to superpests, and its impact on biodiversity. Few of these concerns have been analyzed on strawberry. Asao *et al*. (2003) reported the environmental risk evaluation of a transgenic strawberry with enhanced fungal pathogen tolerance. They concluded that the effects of transgenic strawberry on other plants, radish and spinach, as well as on microflora were not different from that of a non-transgenic strawberry. One of the potential environmental risks of plants engineered with herbicide or pathogen tolerance is the gene escape to wild relatives and the creation of super-weeds. This risk is relatively low in strawberry. Cultivated strawberry lacks significant weedy characteristics and the introduction of pest resistance genes would be unlikely to substantially increase the crop's ability to persist (Rosskopf 1999). Even more, pest resistance genes have been incorporated through conventional breeding for several decades, but increased weediness of strawberry has not been observed. Additionally, as strawberry is an insect-pollinated species, the crossing rate would be much lower than in wind-pollinated species (Watanabe *et al*. 2006). We have evaluated the transfer of the kanamycin resistant gene from transgenic strawberry to wild plants under greenhouse conditions. The transfer rate of the transgene was extremely low, lower than 0.02%, in spite of growing control plants under a high transgenic plant density (unpublished results). Therefore, in the case of transgenic strawberry, the environmental risk of field release is limited and lower than in other crops.

CONCLUSIONS

Efficient transformation protocols have been established for many strawberry commercial cultivars and they have successfully been applied to enhance fruit quality and pathogen tolerance. This last issue still remains as the main challenge of strawberry industry due to lack of natural resistance sources within the *Fragaria* genus. Unfortunately, the negative public acceptance of genetically modify food, especially in Europe, constrains the development of transgenic technology in strawberry. To overcome this negative opinion, strawberry research should be focused on traits that clearly enhance the safety or nutritional value of this crop. Cisgenesis, the genetic transformation with genes from the same or crossable species, would also help to surpass public concerns. Even more, some authors claim to regulate this approach as conventional breeding (Jacobsen and Schouten 2007). Obviously, this will require an extensive exploration of the *Fragaria* genome to avoid the use of foreign genes. Environmental risk assessment as well as nutritional and safety evaluation of transgenic fruits are also research areas that would need further development in the future.

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