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Review of Factors Affecting Organogenesis, Somatic Embryogenesis and *Agrobacterium tumefaciens*-Mediated Transformation of Strawberry

Amjad Masood Husaini[1*] • José A. Mercado[2] • Jaime A. Teixeira da Silva[3] • Jan G. Schaart[4]

[1] Division of Plant Breeding and Genetics, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu & Kashmir-191121, India
[2] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", (IHSM-UMA-CSIC), Departamento de Biología Vegetal, Universidad de Málaga, 29071, Málaga, Spain
[3] Department of Horticultural Science, Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[4] Wageningen UR Plant Breeding, Wageningen University and Research Centre, P.O. Box 16, 6700 AA, Wageningen, The Netherlands

Corresponding author: * **amjadhusaini@yahoo.com, dr.amjadhusaini@hotmail.com**

ABSTRACT

Standardization of an efficient regeneration system for each strawberry genotype is generally an indispensible pre-requisite for the successful development of transgenic plants. In this paper, we review some key factors affecting the regeneration of strawberry plants via adventitious organogenesis or somatic embryogenesis, such as type of explant, growth regulators or dark/light treatments. Since *Agrobacterium tumefaciens*-mediated transformation is the method of choice for strawberry transformation, we review the strategies adopted by different scientists to achieve higher transformation efficiencies and recovery of marker-free transgenic plants. Sufficient *Agrobacterium* cells during cocultivation, an adequate cocultivation period, the use of *vir* gene inducers like acetosyringone, introduction of a pre-selection phase between co-cultivation and selection, and optimum selection pressure, are all important factors to obtain stable transformants. For effective transformation, the antibiotic regime should control bacterial growth without inhibiting the regeneration of plant cells. A general protocol for the *Agrobacterium* transformation of strawberry leaf discs is also described. Finally, we discuss the metrics employed by different researchers for measuring the success of transformation, and highlight the difference between transformation efficiency and transformation percentage.

Keywords: cocultivation, *Fragaria* sp., genetic transformation, *in vitro*, *npt*II gene, regeneration

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BA**, N^6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kan**, Kanamycin; **MS**, Murashige and Skoog medium; **NAA**, α-naphthalene acetic acid; **PGR**, plant growth regulator; **TDZ**, thidiazuron

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INTRODUCTION

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) is an economically important berry crop with immense demand for fresh as well as fruit processing industry, while its wild relative, woodland strawberry (*Fragaria vesca* L.) is of scientific importance due to its small genome size, and genome sequencing project (Shulaev *et al.* 2008). Strawberry breeding programs have been very active in the last few decades. Faedi *et al.* (2002) reported that 463 new cultivars were commercially established from 79 public agencies and 32 private companies during the 1980s and 90s. Nowadays, most strawberry-producing countries have their own breeding programs, both public and private, devoted mainly to develop new cultivars adapted to local conditions. However, *F.* × *ananassa* has a complicated octoploid ($2n=8x=56$)

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genome, and due to genetic limitations associated with high heterozygosity and polyploidy, it has a limited potential for improvement using traditional breeding methods. The application of plant tissue culture and genetic engineering therefore, holds special significance for strawberry improvement (Husaini and Srivastava 2006a). Octoploid strawberry accessions are extremely variable from genotype to genotype, and variation in transformation and regeneration ability is as wide as their agro-morphological characters (Folta and Dhingra 2006). The response to factors affecting genotype-specific regeneration makes standardiza-tion of an efficient regeneration system for each strawberry genotype an indispensible prerequisite for the successful development of transgenic plants using *Agrobacterium*-mediated transformation (Husaini *et al.* 2008).

In this review, we have compared the published literature according to the main variables in the regeneration and transformation processes. First, we highlight the factors for efficient regeneration of complete plantlets under *in vitro* conditions. Second, we highlight the factors influencing *Agrobacterium*-mediated transformation and recovery of transgenic strawberry plants, describing a transformation protocol developed for leaf discs of 'Chandler' that has been successfully used in other genotypes of commercial importance. Next, we discuss the approaches used and progress made in the development of marker-free genetically modified strawberry plants. Finally, we discuss the metrics employed by different researchers for measuring the success of transformation and highlight the differences between them.

MICROPROPAGATION AND *IN VITRO* REGENERATION

Plant tissue culture and regeneration *in vitro* is a complex phenomenon, influenced by a number of genetic and environmental factors. Each species has its own specific requirements for *in vitro* regeneration, and for some recalcitrant genotypes this step is the main bottleneck for their genetic improvement by genetic transformation. Fortunately, strawberry can be easily managed under *in vitro* conditions and efficient protocols for micropropagation and *in vitro* regeneration via adventitious organogenesis or somatic embryogenesis have been developed for many cultivars. The main factors affecting strawberry tissue culture will be discussed in the following sections.

Strawberry micropropagation

Micropropagation of strawberry plants was introduced about three and half decades ago (Boxus 1974), and it is widely used in the USA in commercial propagation of strawberries, as well as in breeding programs (Zimmerman 1981). This technique has been adopted by most European nurseries producing millions of disease-free plants per year (Mohan *et al.* 2005).

The clonal propagation of strawberry provides added advantage for the stable transfer of a single dominant gene for a desired trait into commercially important genotypes without sexual recombination (Husaini and Abdin 2007). Successful shoot proliferation has been obtained in strawberry from single meristems (Boxus 1974), meristem callus (Nishi and Oosawa 1973) and from node culture (Bhatt and Dhar 2000). However, the explant chosen for strawberry micropropagation has been the meristem from runner tips (Sowik *et al.* 2001). Meristems are cultured on a medium containing a high cytokinin concentration, with no or low levels of auxin. This medium promotes axillary budding as the use of cytokinins overcomes apical dominance and enhances the branching of lateral buds from the leaf axis (Debnath 2003; Haddadi *et al.* 2010).

Amongst a vast number of protocols developed for *in vitro* regeneration of strawberry, recently developed protocols enable strawberry micropropagation in a single step where shoot multiplication and rooting takes place in the same culture medium (Debnath 2006; Husaini *et al.* 2008). The use of microcuttings developing both roots and shoots in a medium containing cytokinin is a better choice than multiple shoot proliferation with subsequent rooting of shootlets. In 'Chandler', after induction of somatic embryos on a medium containing thidiazuron (TDZ), embryos successfully germinate and develop small shoots and roots on a medium containing kinetin (Husaini *et al.* 2008).

Factors affecting organogenesis in strawberry

Regeneration via shoot organogenesis has been described in different strawberry cultivars and many scientists have investigated various factors influencing organogenesis (Debnath and Teixeira da Silva 2007). These studies have demonstrated the importance of factors including plant growth regulator (PGR) balance, culture conditions, genotype and explant type on successful plant regeneration (Liu and Sanford 1988; Nehra *et al.* 1989, 1990c; Sorvari *et al.* 1993; Flores *et al.* 1998; Schaart *et al.* 2002; Passey *et al.* 2003; Zhao *et al.* 2004; Qin *et al.* 2005a, 2005b; Husaini and Abdin 2007; Husaini *et al.* 2008). The major factors that influence organogenesis are discussed in the sections that follow.

1. Explant

There are a large number of reports on strawberry shoot organogenesis using a broad range of explants, e.g. leaf disks (Jones *et al.* 1988; Liu and Sanford 1988; Nehra *et al.* 1989, 1990c; Sorvari *et al.* 1993; Flores *et al.* 1998; Passey *et al.* 2003; Qin *et al.* 2005; Debnath 2006; Husaini and Srivastava 2006b; Husaini and Abdin 2007), petioles (Foucault and Letouze 1987; Isac *et al.* 1993; Damiano *et al.* 1997; Popescu *et al.* 1997; Infante *et al.* 1998; Passey *et al.* 2003; Debnath 2006), stems (Graham *et al.* 1995), peduncles (Foucault and Letouze 1987; Lis 1993), stolons (Lis 1993), stipules (Rugini and Orlando 1992; Passey *et al.* 2003), runners (Liu and Sanford 1988), roots (Rugini and Orlando 1992; Passey *et al.* 2003), anthers (Owen and Miller 1996), embryos (Wang *et al.* 1984), sepals (Debnath 2005), and protoplasts (Nyman and Wallin 1988). Most of the work in strawberry regeneration, however, has been achieved using leaf discs and petioles as explants. Leaf tissue has the greatest regeneration capacity of all strawberry plant tissue (Jones *et al.* 1988; Liu and Sanford 1988; Nehra *et al.* 1989, 1990c; Jelenkovic *et al.* 1990; Popescu *et al.* 1997; Passey *et al.* 2003), and shoot regeneration rates using this explant are generally high, although very genotype dependent. **Fig. 1** shows the adventitious regeneration process in a leaf disk. Callus production is also more prolific from leaf tissue on Murashige and Skoog (1962) (MS) medium containing 6-benzyladenine (BA) and indole-3-butyric acid (IBA) (Husaini and Srivastava 2006b). Passey *et al.* (2003) studied adventitious regeneration on seven commercial cultivars of strawberry using leaf disks, petioles, roots, and stipules as explant material. Leaf disks had the highest regeneration rates for all cultivars with greater than 90% of explants producing shoots. Therefore, the leaf disk has been the explant of choice in strawberry transformation studies.

A few studies have evaluated the influence of explant type on transformation efficiencies. James *et al.* (1990a) found that petioles of 'Rapella' were transformed more efficiently than leaf discs. By contrast, this last explant gave better results than stipules in *F. vesca* (Alsheikh *et al.* 2002). Using a different approach, Mathews *et al.* (1995, 1998) observed higher transformation rates when using meristematic sections obtained from the base of *in vitro* proliferating plantlets. However, a high percentage of regenerated plants were chimeras.

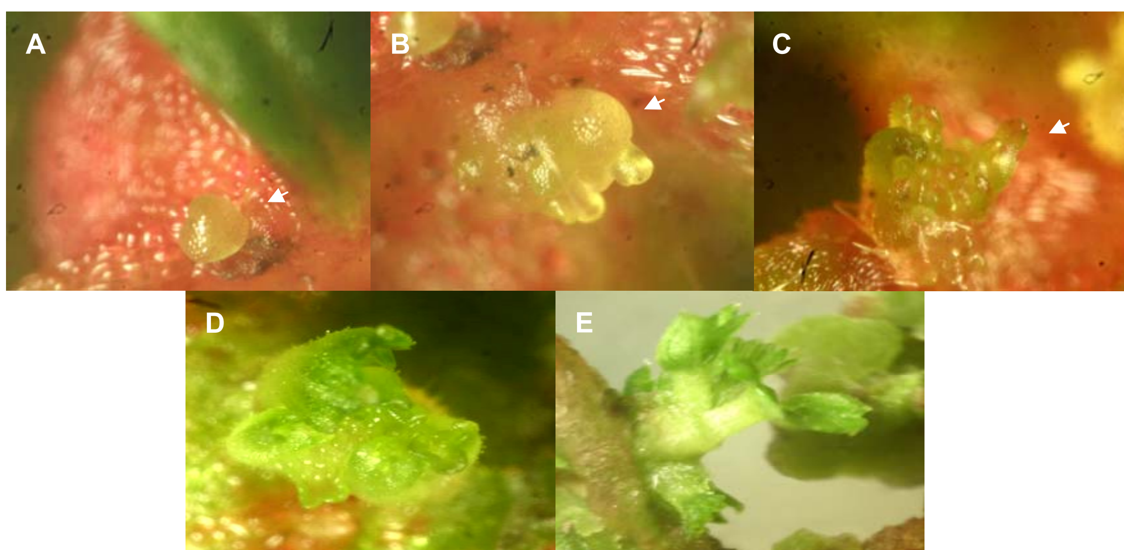

Fig. 1 Direct shoot regeneration in *Fragaria* × *ananassa* Duch. (**A**) Shoot bud initiation. (**B, C, D**) Differentiation of shoot bud. (**E**) Multiple shoot formation.

2. Plant growth regulators

The kind of PGR and the amount used for strawberry regeneration has been highly variable. In general, a combination of auxin and cytokinin is necessary for successful regeneration. Indole-3-acetic acid (IAA) and BA were successfully used by Nehra *et al.* (1989) in 'Redcoat' and by Singh and Pandey (2004) in 'Sweet Charli' and 'Pajaro' cultivars, while IBA and BA gave promising results in 'Hiku', 'Jonsok' (Sorvari *et al.* 1993) and 'Chandler' (Barceló *et al.* 1998; Husaini and Srivastava 2006b). Finstad and Martin (1995) regenerated plants by using 2,4-dichlorophenoxyacetic acid (2,4-D) and BA in 'Totem' and 'Hood', while Qin *et al.* (2005a, 2005b) used TDZ and IBA in 'Toyonoka'.

In recent years, there has been an increased interest in the use of the cytokinin TDZ in strawberry regeneration. Many studies have revealed that TDZ is very effective promoting shoot regeneration in strawberry leaf disks (Nyman and Wallin 1992; Sutter *et al.* 1997; Hammoudeh *et al.* 1998; Flores *et al.* 1998; Schaart *et al.* 2002; Passey *et al.* 2003; Zhao *et al.* 2004; Qin *et al.* 2005a, 2005b; Landi and Mezzetti 2006; Husaini and Abdin 2007), sepals and petioles (Debnath 2008, 2009). The variability in the regeneration percentages obtained in these reports is due to the use of different concentrations of TDZ and different strawberry cultivars, indicating that each genotype has specific requirements that are vital for regeneration.

3. Light/dark period

The problem of darkening of culture medium of *in vitro* cultured strawberry explants is well-known and it is attributed to phenolic compounds exuding from these tissues. This process is initiated by browning of the surface of plant tissues due to the oxidation of phenolic compounds resulting in the formation of quinines which are highly reactive and toxic to plant tissue (Taji and Williams 1996). Dark incubation reduces tissue browning by arresting the enzymatic activity responsible for tissue oxidation (George 1993; Titov *et al.* 2006). In strawberry, incubation of leaf explants in the dark decreases browning of the culture medium (Nehra *et al.* 1989; Rugini and Orlando 1992; Blando *et al.* 1993; Popescu *et al.* 1997; Barceló *et al.* 1998; Husaini and Abdin 2007). Besides this, it seems that a dark treatment for several weeks (Liu and Sanford 1988; Barceló *et al.* 1998; Husaini and Abdin 2007) or even continuous darkness incubation (Landi and Mezzetti 2006), depending on the cultivar, enhances organogenesis in strawberry leaf explants. In 'Chandler', a comparison of photoperiods (24-, 16-, 12-h) used for incubation of strawberry leaf disks revealed that a 16-h photoperiod was the optimum for shoot organogenesis (Husaini and Abdin 2007).

Regarding light intensity, Barceló *et al.* (1998) and Husaini and Abdin (2007) found that 40-48 μmol m^{-2} s^{-1} was optimal for regeneration of 'Chandler' leaf explants. However, a study by Nehra *et al.* (1990c) in which identical sets of cultures of 'Redcoat' were incubated at 12.5 and 65.5 μmol m^{-2} s^{-1} revealed that calli from *in vitro* leaves did not form shoots under high light intensity on any of the culture media, but at low light intensity some calli developed into shoots.

Factors affecting somatic embryogenesis in strawberry

In the plant body, all cells have specific functions to play and cells dedifferentiate prior to becoming competent to respond to the new signals. *In vitro* plant organization involves a two-step process where first, a cell or a tissue acquires developmental competency (totipotency) and subsequently is determined for one structure or another by environmental factors (Decout *et al.* 1994). Somatic embryogenesis is a process by which the somatic cells undergo a developmental process similar to the development of zygotic embryos (Williams and Maheshwaran 1986) and it is considered as an extreme response of somatic plant cells

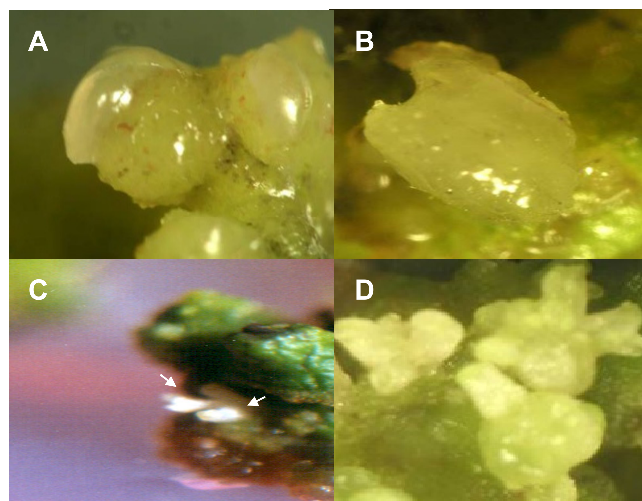

Fig. 2 Direct somatic embryogenesis in *Fragaria* × *ananassa* Duch. (**A**) Globular embryos on leaf epidermis. (**B**) Heart-shaped embryo. (**C**) Advanced cotyledonary embryos. (**D**) Embryos germinating.

towards specific stress conditions. Only a few studies have so far focused on somatic embryogenesis in strawberry, primarily showcasing the importance of PGRs and growth media (Wang *et al.* 1984; Lis 1987; Donnoli *et al.* 2001; Biswas *et al.* 2007; Husaini and Abdin 2007; Husaini *et al.* 2008; Kordestani and Karami 2008; reviewed by Debnath and Teixeira da Silva 2007). The next sections review the main factors influencing somatic embryogenesis in strawberry; this process is illustrated in **Fig. 2**.

1. Plant growth regulators and culture media

Several culture media and PGR combinations have been used to achieve somatic embryogenesis in strawberry. According to Wang *et al.* (1984) the most effective medium for inducing strawberry somatic embryos contained 2,4-D (22.62 μM), BA (2.22 μM) and casein hydrolysate (500 mg l^{-1}), while Lis (1987) reported the formation of adventitious buds and somatic embryos using the medium of Lee and de Fossard (1977). Biswas *et al.* (2007) found that α-naphthalene acetic acid (NAA) at 21.5 μM was the most efficient for leaf callus induction, and that MS medium supplemented with 4.5 μM 2,4-D, 2.2 μM BA and 50% proline was the best medium for somatic embryogenesis. Kordestani and Karami (2008) reported the induction of somatic embryogenesis in leaves from 'Camarosa' and 'Selva' cultured on MS medium supplemented with 8.3 μM picloran. In this study, globular embryos were transferred to a hormone free medium for maturation, and later converted to plantlets after transfering cotyledonal embryos to MS supplemented with gibberellic acid. Husaini and Abdin (2007) for the first time could achieve shoot regeneration in strawberry simultaneously through both somatic embryogenesis and shoot bud formation. In this study, leaf explants were cultured in MS medium supplemented with a relatively high TDZ concentration (18.16 μM). Based on this study, more recently, Husaini *et al.* (2008) developed a reliable and highly efficient somatic embryogenesis system for 'Chandler' and examined the effect of temperature on the induction and maintenance of somatic embryos.

2. Light and photoperiod

Light is known to affect somatic embryogenesis through its effect on induction (Verhagen and Wann 1989) and on some morphological characteristics of differentiated somatic embryos (Halperin 1966; Ammirato and Steward 1971). Despite these powerful effects of light, little attention has been devoted to its role in *in vitro* culture (Torné *et al.* 2001) and particularly somatic embryogenesis. Photoperiod has been implicated in the regulation of cytokinin levels (Forsline and Langille 1975) as well as in photoconversion of phytochromes (Torné *et al.* 1996). In strawberry, a negative effect of light on somatic embryo induction has been reported in 'Clea' (Donnoli *et al.* 2001) and the clone pbgel-2000 (Biswas *et al.* 2007). Similarly, in 'Chandler', a dark treatment significantly increased the number of somatic embryos in the leaf explants cultured on 18.16 μM TDZ and later incubated under a 16-h photoperiod (Husaini and Abdin 2007). The response to photoperiod, however, can be modified by other environmental factors, since explants subjected to a chilling treatment showed an optimal photoperiod of 12-h instead of the 16-h treatment (Husaini and Abdin 2007).

3. Chilling

High or low temperature stress can stimulate somatic embryogenesis. Heat stress is effective for the induction of pollen embryos in canola (Pechan *et al.* 1991) while cold stress increases the embryogenic potential of strawberry (Husaini and Abdin 2007). Husaini *et al.* (2008) clearly demonstrated that the concentration of TDZ is the primary factor responsible for induction of somatic embryogenesis in strawberry, while incubation at a temperature regime of 10 ± 1°C had a complementary effect on increasing the number of somatic embryos per explant. Chilling treatment might have some effect on the microtubule network of the cytoskeleton in strawberry as has been reported in chicory, where it was postulated that low temperature might induce a different behaviour of the cytoskeleton leading to different morphogenesis (Decout *et al.* 1994).

FACTORS AFFECTING TRANSFORMATION AND REGENERATION OF TRANSGENIC PLANTS

During the last two decades a number of studies with the objective of standardizing transformation protocols for different strawberry cultivars were undertaken (reviewed in Folta and Dhingra 2006; Husaini and Srivastava 2006a; Mercado *et al.* 2005, 2007a; Quesada *et al.* 2007; Qin *et al.* 2008). Most of these studies used *Agrobacterium tumefaciens* infection as the system for gene delivery. It is well-known that a rigorous transformation process can reduce the regeneration capacity of a strawberry tissue (leaf) drastically, and may slash it from approximately 95% to 1-6% (Passey *et al.* 2003). In *Agrobacterium*-mediated transformation, a sufficient quantity of bacteria during cocultivation, a long enough cocultivation period, use of *vir* gene inducers like acetosyringone, and stringent selection pressure are important to obtain stable transformants. Furthermore, for effective transformation, the antibiotic regime should control bacterial overgrowth without inhibiting the regeneration of the plant cells (Graham *et al.* 1995; Alsheikh *et al.* 2002; Qin *et al.* 2011). It is therefore appropriate to review the effect of such factors on genetic transformation of strawberry.

Robust regeneration system and efficient *Agrobacterium* strain

Establishment of a regeneration system for efficient recovery of transformed cells following agroinfection is of utmost importance in *Agrobacterium*-mediated transformation of strawberry (Husaini and Srivastava 2006b; Debnath and Teixeira da Silva 2007; Husaini *et al.* 2008). Use of a high efficiency regeneration system greatly enhances induction of shoot organogenesis from transformed cells. The better a regeneration system, the greater are the chances of successful recovery of transgenic plants (Husaini 2010). Even on the same selection medium, the efficiency of shoot production varies with the strain of *A. tumefaciens* and binary vector used. Most binary vectors used to transform strawberry are derived from pBIN19 (Bevan 1984) and contain the neomycin phosphotransferase-II (*npt*II) gene for kanamycin (Kan) selection of transgenic shoots (Mercado *et al.* 2007a). As a combination, *Agrobacterium* strain LBA4404 and gene construct pBI121 have been used extensively in strawberry transformation of 'Rapella' (James *et al.* 1990ab), 'Melody', 'Rhapsody', 'Symphony' (Graham *et al.* 1995), 'Chandler' (Barceló *et al.* 1998; Husaini and Srivastava 2006b) and *F. vesca* (El Mansouri *et al.* 1996; Alsheikh *et al.* 2002). In addition, *Agrobacterium* strain GV2260 has also been successfully used for genetic transformation of 'Marmolada onebor' (Martinelli *et al.* 1997), 'Selekta' (Du-Plessis *et al.* 1997), *Fragaria* × *ananassa* breeding selection 'AN 93.231.53' (Mezzetti *et al.* 2004) and 'Chandler' (Husaini and Abdin 2008a, 2008b). Finally, the supervirulent *Agrobacterium* strain Agl0 (Lazo *et al.* 1991) and derivatives EHA101 and EHA105 (Hood *et al.* 1986) have been successfully used in transformation of 'Tristan', 'Totem' (Matthews *et al.* 1995), Elsanta (Puite and Schaart 1998), 'Polka', 'Gariguette', breeding line 88312 (Schaart *et al.* 2002) and 'Calypso' (Schaart *et al.* 2011a).

Antibiotics, regeneration and selection

Post-agroinfection exposure of explant tissues to two classes of antibiotics, one for selection of transformed cells and other for eliminating *Agrobacterium*, is optimized to effect a compromise between producing transgenics and

screening-out escapes. Among the antibiotics used for selection, Kan is the most widely used for transformation studies in strawberry, while some have successfully used hygromycin (Nyman and Wallin 1992; Mathews *et al.* 1995; Oosumi *et al.* 2006), geneticin (Mathews *et al.* 1995), and the herbicide phosphinothricin (Wang *et al.* 2004; Folta *et al.* 2006). The concentration of Kan in the selection media has a significant effect on transformation efficiencies. Shoot regeneration from leaf disks is impaired at Kan concentrations as low as 10 mg l^{-1} (El Mansouri *et al.* 1996; Barceló *et al.* 1998; Gruchala *et al.* 2004a) and higher Kan concentrations in the selection medium significantly reduce shoot regeneration (Alsheikh *et al.* 2002; Husaini 2010). The concentration of Kan used for transgenic selection varies with cultivar, explant type and the selection procedure employed. For example, Nehra *et al.* (1990a, 1990b), in 'Redcoat' leaf explants, used a Kan concentration of 50 mg l^{-1} during the first 4 weeks of culture and then transferred the explants to 25 mg l^{-1} Kan. Similarly, Graham *et al.* (1995) cultured stem sections of 'Melody', 'Rhapsody' and 'Symphony' at 20 mg l^{-1} Kan for 5 days and later at 10 mg l^{-1}. Others have used an opposite selection procedure; e.g., Husaini and Abdin (2008a) and Husaini (2010) used higher Kan concentration (50 mg l^{-1}) in the beginning of the selection phase, just after a 5 days pre-selection period, and later reduced Kan to 25 mg l^{-1}. In other cases, constant selection pressure was applied, e.g. 'Rapella' petioles were cultured at 25 mg l^{-1} (James *et al.* 1990b), 'Chandler' leaf at 25 mg l^{-1} (Barceló *et al.* 1998; Cordero de Mesa *et al.* 2000), 'Teodora' and 'Egla' stipules at 50 mg l^{-1} (Monticelli *et al.* 2002). Interestingly, in 'Calypso', Kan was used at 150 mg l^{-1} for selection of transgenic plants (Schaart *et al.* 2004), indicating that some genotypes may be very resistant to the compound.

In some strawberry transformation studies the use of Kan has been related to the risk of formation of shoots containing transgenic and non-transgenic sections (chimeras) (Mathews *et al.* 1998; Shestibratov and Dolgov 2005), especially when using stipules (Monticelli *et al.* 2002; Chalavi *et al.* 2003) or meristematic sections of *in vitro* plants (Mathews *et al.* 1998) as explants. This is probably due to high antibiotic tolerance of the particular cultivar, since non-transformed shoots (control) were also able to grow and proliferate at the Kan concentration used for selection (Mercado *et al.* 2007a). Two methods are employed to induce transgenic shoots on selection medium, one in which the concentrations of Kan are kept constant (non-iterative method), the other where its levels are increased gradually during subculture (iterative method). The iterative method has been shown to inhibit the development of chimeric plants (Mathews *et al.* 1998; Houde *et al.* 2004). Various antibiotics used to control *Agrobacterium* growth exhibit phytotoxicity, especially at high concentrations. The use of carbencillin to control *Agrobacterium* after transformation of strawberry leaf explants (cultivar 'Totem') resulted in stunted top and root growth of plantlets while with timentin [a mixture of ticarcillin (96%) and clavulanic acid (4%)] the regenerated plantlets showed vigorous, healthy top and root growth (Finstad and Martin 1995). In contrast, in octaploid strawberry genotype 'LF9' timentin, though found to be effective in curbing *Agrobacterium* growth, slowed its growth and differentiation slightly (Folta *et al.* 2006). Alsheikh *et al.* (2002) compared regeneration of *F. vesca* and *F. vesca semperflorens* in the presence of different concentrations of carbenicillin, cefotaxime, and cefoxitin, from 10 to 500 mg l^{-1}, and concluded that amongst these antibiotics, carbenicillin was the least phytotoxic. Moreover, phytotoxicity varied with the type of explant used, petioles being more sensitive to antibiotic toxicity than leaf disks. Hanhineva and Karenlampi (2007) found that cefotaxime inhibited shoot regeneration in cv. 'Jonsok', especially at a high concentration (500 mg l^{-1}). These results show that the interaction of antibiotic with plant species is genotype dependent, and that variations occur because of *Agrobacterium* strain, explant type and the cultivar under study.

A combination of agrocidal antibiotics with a synergistic effect has proven less phytotoxic and better in eliminating *Agrobacterium* than when used in isolation at identical concentrations (Tanprasert and Reed 1998; Husaini 2010). Husaini (2010) showed that a combination of timentin and cefotaxime at 250 mg l^{-1} each is less phytotoxic to leaf disks of 'Chandler' than the use of either of these antibiotics alone at higher concentrations (500 mg l^{-1}).

Pre-culture (pre-incubation)

Prior to inoculation with *Agrobacterium*, explants are sometimes incubated on a regeneration medium for a period of 1-10 days, allowing these explants to adjust to the regeneration media. This practice of pre-culturing explants has been shown to be beneficial in most cases (Sorvari *et al.* 1993; El Mansouri *et al.* 1996; Asao *et al.* 1997; Barceló *et al.* 1998; Cordero de Mesa *et al.* 2000; Alsheikh *et al.* 2002; Husaini 2010). Pre-culturing improves transformation percentage, probably by increasing the number of plant cells competent for regeneration and transgene integration (Birch 1997).

Co-cultivation and *vir* inducer treatments

Cocultivation of explant with genetically engineered *Agrobacterium* is a crucial step in gene transfer, as an excessive number of bacteria imposes stress on plant cells, negatively affecting their regeneration potential, and a lower number reduces the frequency of T-DNA transfer (Montoro *et al.* 2003). Increased co-cultivation period can enhance transfection events but may also cause tissue necrosis due to related stress. Co-cultivation period varies between 15 min (Nehra *et al.* 1990b) and 2 h (Mathews *et al.* 1998); however, most strawberry researchers advocate co-cultivation for a duration between 24 and 72 h in the dark (Zhang and Wang 2005; Folta and Dhingra 2006; Husaini 2010).

It is well known that phenolics, like acetosyringone, and other bacterial culture factors such as low pH, increase *Agrobacterium* virulence by the activation of *vir* genes (Karami *et al.* 2009). In most strawberry cultivars the addition of acetosyringone during preculture and cocultivation showed a synergistic effect on *Agrobacterium*-mediated transformation, increasing the number of transformed cells in target tissues (James *et al.* 1993; Alsheikh *et al.* 2002; Gruchala *et al.* 2004a; Husaini 2010). However, there is huge variation in the degree of responses between these studies, which may be due to extreme genotype dependence and variability in regeneration and transformation rates for different cultivars (Alsheikh *et al.* 2002; Quesada *et al.* 2007) or to suppression of virulence in some strain/plant species interactions (Godwin and Todd 1991).

Pre-selection

Selective agents like Kan have been shown to interfere with the regeneration of transformants (van Wordragen 1992; Husaini 2010). A delay period of 2 to 10 days before challenging the infected cells to selective agents (called the preselection phase) is sometimes introduced to allow the transformed cells to recover from the infection process and to express the selectable marker gene (Alsheikh *et al.* 2002; Zhao *et al.* 2004). Leaf disks inoculated with *Agrobacterium* regenerate shoots at a low frequency when subjected to selection pressure immediately after co-cultivation, while the introduction of a short preselection phase significantly increases the percentage of leaf disks regenerating shoots (Nehra *et al.* 1990a, 1990b; Alsheikh *et al.* 2002). In 'Chandler', the percentage of explants regenerating shoots increased by almost 6-fold with a 5-day preselection phase, from 0.5% (no preselection) to 3.1% (5-days preselection) (Husaini 2010). By contrast, the absence of selection during a long period after explant infection, e.g. 3 weeks, can reduce transformation efficiency (Mathews *et al.* 1995).

One effective mechanism to reduce damage from stress

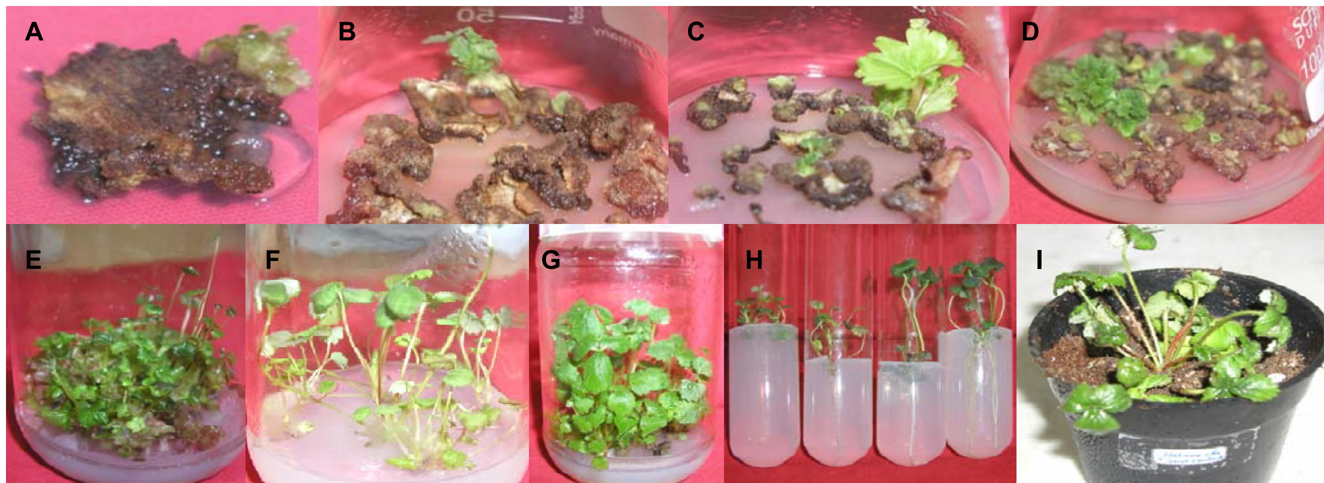

Fig. 3 Development of transgenic plants of *Fragaria* × *ananassa* Duch. (**A**) Shoot initiation. (**B, C, D**) Differentiation of shoots. (**E, F, G**) Multiple shoot formation and elongation. (**H**) Root formation. (**I**) Acclimatization in pot.

is the accumulation of high intracellular levels of trehalose (Crowe *et al.* 1984; Drennan *et al.* 1993; Goddijn and van Dun 1999). Recently, Husaini (2010) used a specific trehalase inhibitor, validamycin A, in pre-selection culture medium to reduce the effect of stress on transformed cells imposed by the process of transformation and to facilitate the recovery of Kan-resistant putative transformants. The addition of validamycin A in the preselection medium resulted in a two-fold (from 3.1 to 7.4%) increase in the average percentage of leaf disks regenerating shoots on selection medium. As the plant trehalose biosynthesis pathway is tightly regulated by multiple stress signals (Drennan *et al.* 1993; Pramanik and Imai 2005), the addition of validamycin A probably reduces the 'transformation-stress' on the cells caused due to agroinfection.

A protocol for strawberry transformation

The following section describes a general protocol for the *Agrobacterium*-mediated transformation of strawberry leaf disks. This method was developed for the transformation of 'Chandler' (Barceló *et al.* 1998; Husaini 2010) but, with modifications, has been used to transform other commercial cultivars, such as 'Camarosa', 'Andana' or 'Carisma'. This protocol uses disarmed *A. tumefaciens* strains, LBA4404 or GV2260, containing a binary vector harboring the Kan resistance gene *npt*II, driven by the nopaline synthase promoter, for selection. Leaf disks for *Agrobacterium* inoculation are obtained from stocks of plants micropropagated *in vitro* derived from the culture of runner tips of virus-free plants growing in the greenhouse. Adventitious regeneration medium should be adjusted for each specific cultivar. A medium containing N30K macroelements supplemented with 2.46 µM IBA and 8.8 µM BA has been found to be optimal for 'Chandler' and 'Camarosa' regeneration (Barceló *et al.* 1998; Mercado *et al.* 2007b). Husaini (2010), on the other hand, used MS supplemented with 18.1 µM TDZ.

1. Explant preculture

Green leaves from *in vitro* stocks are cut into small pieces (0.5 cm^2), and precultured on shoot regeneration medium in the dark for 7 days.

2. Growth of Agrobacterium culture

Agrobacterium is grown at 28°C in Luria Broth supplemented with appropriate antibiotics for bacterial and binary plasmid selection, 200 mg l^{-1} streptomycin for LBA4404 and 75 mg l^{-1} rifampicin for GV2260 strain. After 24 h, bacterial cultures are centrifuged at 5000 rpm and the pellet resuspended in 25 ml MS liquid medium with the addition of filter sterilized acetosyringone, 100 µM. The suspension is grown for 3-4 h at 28°C until it attains an optical density of 1 at 600 nm (approximately 10^8 cells/ml).

3. Inoculation and co-cultivation of explants

Precultured leaf explants are immersed in the *Agrobacterium* suspension for 25 min with gentle agitation. Afterwards, explants are blotted dry on sterile filter paper and cultured in the regeneration medium for 2 days, at 25°C in the dark.

4. Selection of transformed plants

After co-cultivation, explants are sequentially washed with sterile water and a solution of cefotaxime and timentin (both at 250 mg l^{-1}) for 15 min. Then, explants are blotted dry with sterile filter paper and cultured in the pre-selection medium (regeneration medium supplemented with cefotaxime and timentin, both at 250 mg l^{-1}, and 100 µM validamycin A) for 5 days. Afterwards, explants are transferred to selection medium (pre-selection medium supplemented with 50 mg l^{-1} Kan), and subcultured every 4 weeks onto fresh medium. Selection is accomplished at 25°C with a 16-h photoperiod of 40 µmol m^{-2} s^{-1}. Regenerated shoots start to appear after 4-8 weeks of culture in the selection medium, usually in form of clusters composed of several shoots. Then, a single shoot per explant is isolated and micropropagated in the appropriate medium supplemented with Kan at 25 mg l^{-1}. In the case of 'Chandler' and 'Camarosa', N30K medium (Margara 1984) supplemented with 2.21 µM kinetin can be used for shoot elongation and rooting. Plants with shoots 5-6 cm in length can be acclimated to *ex vitro* conditions following standard techniques (López-Aranda *et al*. 1994).

Using this protocol, a transformation rate varying between 4 and 10%, estimated as the number of independent transgenic plants recovered from 100 inoculated leaf disks, can be obtained in a period of 16-20 weeks. **Fig. 3** shows the development of transgenic plants in 'Chandler' employing this transformation method. The differences in protocols between the Husaini (2010) and the Barceló *et al*. (1998) transformation protocols are highlighted in **Tables 1** and **2**.

Production of marker-free genetically modified strawberry plants

Public concerns on the issue of the environmental and food safety of genetically modified plants have led to a demand for technologies allowing the production of transgenic plants without selectable (antibiotic resistance) markers. In strawberry, marker-free genetically modified plants have

Table 1 Major differences in *Agrobacterium tumefaciens* mediated transformation protocols of *Fragaria x ananassa* Duch 'Chandler'.

Parameter	Barceló *et al.* 1998	Husaini 2010
Source of explant	Variable	20-day-old plantlets maintained on MS salts + B_5 Vit + glucose (2%) + agar (0.9%) + kinetin (1 mg/l)
Shoot regeneration medium	Lopez Aranda *et al.* 1994	Murashige and Skoog 1962 + B_5 Vit + 2% glucose*
Efficiency of regeneration system (%)	66.7	100
Agrobacterium tumefaciens strain	LBA 4404	GV 2260
Binary vector	pBI121	pBinAR
Acetosyringone (µM)	0	100
Co-cultivation duration (h)	72	72
Kanamycin in selection medium (mg/l)	25	50 and 25
Agrobactericidal antibiotics (mg/l)	Carbencillin 500	Cefotaxime 250 + Timentin 250
Osmoprotectant (µM)	0	Validamycin A 100
Pre-culture/pre-incubation duration (days)	3, 10	7
Pre-selection (days)	0	5
Transformation % based on number of explants regenerating shoots on kanamycin	4.2	10

* Also see **Table 2**

Table 2 Media used for transformation and recovery of transgenic plants of cultivar 'Chandler' (according to Husaini 2010).

Medium	Components
MS liquid medium MSL	MS salts and vitamins + 3% sucrose
Regeneration medium R_M	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ
Shoot elongation medium SM_I	MS salts + B_5 vitamins + 2% sucrose + 1% glucose + 0.1 mg l^{-1} BA + 0.05 mg l^{-1} Kn + 2 mg l^{-1} GA_3
Pre-selection regeneration medium PS_LM_{IA}	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ + 500 µg ml^{-1} Cefotaxime
PS_LM_{IB}	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ + 500 µg ml^{-1} Timentin
PS_LM_{IC}	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ + 250 µg ml^{-1} Cefotaxime + 250 µg ml^{-1} Timentin
Selective regeneration medium S_LM_{IA}	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ + 500 µg ml^{-1} Cefotaxime + 50 µg ml^{-1} Kanamycin
S_LM_{IB}	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ + 500 µg ml^{-1} Timentin + 50 µg ml^{-1} Kanamycin
S_LM_{IC}	MS salts + B_5 vitamins + 2% glucose + 4 mg l^{-1} TDZ + 250 µg ml^{-1} Cefotaxime + 250 µg ml^{-1} Timentin + 50 µg ml^{-1} Kan
Selective shoot elongation medium S_LM_{II}	MS salts + B_5 vitamins + 2% sucrose + 1% glucose + 0.1 mg l^{-1} BA + 0.05 mg l^{-1} Kn + 2 mg l^{-1} GA_3 + 25 µg ml^{-1} Kan
S_LM_{III}	MS salts + B_5 vitamins + 2% sucrose + 1% glucose + 1.0 mg l^{-1} Kn + 25 µg ml^{-1} Kan
Root induction medium RI_M	MS salts + B_5 vitamins + 2% sucrose + 1% glucose + 1.0 mg l^{-1} Kn

been produced using a method that employs site-specific recombination-mediated excision of the gene originally used for selection of transgenic plants. This method, described by Schaart *et al.* (2004, 2010) uses the site-specific recombination system R/Rs from *Zygosaccharomyces rouxii*, in which activity of the recombinase protein was directly regulated by a chemical inducer. The recombination-induced elimination of undesired sequences was combined with a negative selection step. This step allows to select against failed or incomplete marker elimination, using a negative selectable marker, the *Escherichia coli* cytosine deaminase (*cod*A) gene. This is a conditionally lethal dominant gene encoding an enzyme that converts the non-toxic 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU). The method was tested in strawberry cv 'Calypso' because of its superior regeneration and transformation capacity (Passey *et al.* 2003) and using the test-vector pRCNG (Schaart *et al.* 2004). This test vector has a promoterless *gus* reporter gene which will be combined with a CaMV35S promoter following removal of marker gene sequences which separate both promoter and *gus* gene. So, the *gus*-reporter could be used to monitor recombination events in pRCNG- transformed plants. At first, kanamycin resistant strawberry plants were produced using a standard transformation protocol and selection on 150 mg l^{-1} kanamycin. In a secondary step, leaf explants from the transgenic strawberry plants were subjected to an overnight dexametasone (dex) treatment for induction of the R recombinase activity and subsequently shoots were regenerated from the dex-treated leaf explants using 5-FC as a negative selection agent. This resulted in a high proportion of transgenic plants from which the selectable marker sequences had been removed (Schaart *et al.* 2004). An adapted version of the pRCNG-vector, pMF1 (Schaart *et al.* 2010) which lacks the *gus* reporter gene, and which was equipped with a multiple cloning site, was used to produce intragenic strawberry plants (Schaart *et al.* 2011a). In these plants, a strawberry polygalactronase inhibiting protein gene (*FaPGIP*) (Mehli *et al.* 2004) which was combined with the strong and ripe fruit-specific regulatory elements (promoter and terminator) of a strawberry expansin gene (*FaExp2*) (Schaart *et al.* 2011b) was introduced. Selection of kanamycin resistant plants and subsequent elimination of the marker genes resulted in strawberry plants in which only gene elements originating from strawberry itself were present. Therefore these plants are called intragenic, rather than transgenic. A derived version of pMF1, pHUGE was produced and used to transfer large genomic DNA fragments to the strawberry plant genome (personally communicated by Dr. A. Untergasser and Dr. R Geurts, Wageningen University; results to be published elsewhere). This vector has the vector backbone of pYLTAC7 (Liu *et al.* 1999) and has Gateway cloning sites to facilitate cloning of large DNA fragments. Using the pHUGE vector for transformation of the strawberry cultivar 'Calypso' 33 transgenic strawberry plants could be obtained of which 55% contained a complete integrated T-DNA fragment of 72 or 74 kbp. Subsequent dexamethasone treatment of leaf explants of these plants followed by secondary regeneration removed marker sequences in 80% of these plants. These results demonstrate the possibility of transferring large DNA fragments into strawberry genome in an efficient way, and allow the introduction of complete BAC cloned sequences, without the need to subclone candidate genes from these BAC clones. Therefore pHUGE may be an interesting tool to perform functional analysis of strawberry genes in their chromosomal context in strawberry.

ESTIMATING TRANSFORMATION SUCCESS

There is a wide difference in the methods employed for estimating regeneration and transformation efficiencies. Most strawberry transformation experiments have been performed using leaf disks as explants, and transformation rate

Table 3 Different ways of calculating transformation success in strawberry transformation.

Formula $^{\$}$	Description of formula	Remarks	References
$(NS_{PT} \div NE_{Ai}) \times 100$	Number of putative transgenic shoots regenerated on selection medium (usually after 8 weeks) ÷ Number of explants agro-infected	This formula can be used at early stages of regeneration but may include escapes too.	Husaini 2010
$(NS_{PT} \div NE_{SM}) \times 100$	Number of putative transgenic shoots regenerated on selection medium (usually after 8 weeks) ÷ Number of explants put on selection medium	It ignores the positive/negative effect of pre-selection strategy (pre-culture, pre-selection, Agro-infection) on transformation	Nehra *et al.* 1990a, 1990b
$(NE_{SPT} \div NE_{Ai}) \times 100$	Number of explants regenerating putative transgenic shoots on selection medium (usually after 8 weeks) ÷ Number of explants agro-infected	It ignores multiple transformation events occurring on the same explant but at separate loci.	Zhao *et al.* 2004
$(NS_{PCR} \div NE_{Ai}) \times 100$	Number of PCR confirmed transgenic shoots regenerated on selection medium (usually after 8 weeks) ÷ Number of explants agro-infected	Most accurate formula, but can be used at later stage when sufficient tissue material becomes available for DNA isolation/PCR. However, since some transformants perish in various stages of development, such transformation events are not taken into account.	Husaini 2010
$(NS_{PT} \div NE_{Ai}) \times (NE_{nsm} \div NS_{nsm}) \times 100$	[Number of putative transgenic shoots regenerated on selection medium (usually after 8 weeks) ÷ Number of explants agro-infected] × [Number of explants cultured on non-selective (kanamycin-free) medium ÷ Number of shoots regenerated on non-selective medium]	Technically this is the most appropriate formula for describing transformation 'efficiency', as it compares the relative regeneration capacities of agro-infected and normal (non agro-infected) explants.	Gruchala *et al.* 2004a, 2004b

$^{\$}$ **N** stands for 'Number', **S** for 'shoots' and **E** for 'explants'. $\mathbf{S_{PT}}$ means 'putative transgenic shoots', $\mathbf{E_{Ai}}$ 'agro-infected explants', $\mathbf{E_{SM}}$ 'explants on selection medium', $\mathbf{E_{SPT}}$ 'explants regenerating putative transgenic shoots', $\mathbf{S_{PCR}}$ 'shoots confirmed as transgenic using PCR', $\mathbf{E_{nsm}}$ 'explants on non-selective medium', $\mathbf{S_{nsm}}$ 'shoots regenerated on non-selective medium'.

is based on the number of shoots regenerated per 100 *Agrobacterium* inoculated explants or explants cultured on selection medium. However, each researcher uses his own metric to describe the success of transformation. For example, Gruchala *et al.* (2004b) analyzed 25 strawberry cultivars to select genotypes most suitable for transformation and expressed transformation/regeneration efficiency as the 'transformant number per 100 explants', varying this value between 3 to 9.5, depending on the cultivar. Zhao *et al.* (2004) used the term 'transformation rate' to express transformation success and calculated it as the percentage of 'explants' that regenerated shoots on selection medium after 8 weeks. However, a closer examination reveals that actually transformation success was calculated as a percentage of 'putative transgenic shoots' on selection medium. In the study (Zhao *et al.* 2004), transformation rates varied between 68% in diploid strawberry to 10% in octoploid 'Hecker'.

The number of independent shoots generated per explant is usually referred to as 'regeneration efficiency', while the percentage of 'explants' that produce a transgenic shoot is referred as 'transformation efficiency' (Folta and Dhingra 2006). However, this definition leads to many different formulae (**Table 3**). Formulae 1 to 4 (from the top downwards) in **Table 3** give more weight to the regeneration system used, and hence do not reflect the actual transformation efficiency. These formulae actually aim at calculating the 'number of transformation events' that 'successfully regenerate shoots/plantlets' after application of an appropriate 'selection pressure', and assume that 'every single shoot' represents a 'unique transformation event'. This assumption may however not be correct, because strawberry leaves may regenerate multiple shoots (clusters or colony) per explant per initiation site, resulting in transformation percentage higher than 100%.

The terms 'transformation efficiency' and 'transformation percentage' are not synonymous (Husaini 2010). The former describes the number of transgenic shoots that arise on selection medium as compared with the number of regeneration events that occur in the absence of selection (**Table 3**, formula 5). On the other hand, reporting 'transformation efficiency' as the number of transformants per explant distorts the representation, since Oosumi *et al.* (2006) and Folta *et al.* (2006) reported transformation efficiencies greater than 100%. This metric simply means that each explant produced at least one transgenic shoot. Actually, the transformation efficiency described by Folta *et al.* (2006) is quite low (1–3%). In our opinion there is an objection to reporting 'transformation efficiency' as greater than 100% as mathematically it is incorrect to have efficiencies greater than '1' i.e. 100%. However, transformation percentage of greater than 100% is quite possible especially because each leaf explant can regenerate multiple shoots/shoot clusters in strawberry. When calculating transformation percentage we actually aim to calculate the 'number of transformation events' that 'successfully regenerate shoots/plantlets' when exposed to appropriate selection pressure. Transformation efficiency reports the 'relative' regeneration capacities of agroinfected and control explants, while transformation percentage measures the 'success' in recovering transgenic shoots only (Husaini 2010). Furthermore, as described by Husaini (2010), the parameters used to calculate transformation 'percentage' are extremely important because, based on the method of calculation, different values for transformation percentages can be derived.

FUTURE PERSPECTIVES

Since the pioneering works of Nehra *et al.* (1990b) and James *et al.* (1990a), who for the first time described genetic transformation in strawberry, many protocols have been developed for the *Agrobacterium* transformation of cultivated and wild strawberry. Even more, these procedures have been used to improve important traits, such as fruit quality (Jiménez-Bermúdez *et al.* 2002; Quesada *et al.* 2009), fruit production (Mezzetti *et al.* 2004), fungal resistance (Schestibratov and Dolgov 2005; Vellicce *et al.* 2006) or abiotic tolerance (Houde *et al.* 2004; Husaini and Abdin 2008a). However, *in vitro* regeneration and transformation of strawberry is still far from be a routine technique. A robust regeneration system is an indispensable prerequisite for the success of genetic transformation, and genotype appears as the main factor determining the response of plant tissue to its *in vitro* culture. This dependence on the regeneration system makes transformation efficiencies highly variable among the different transformation studies performed, even when using the same strawberry genotype. The search for novel transformation systems, more efficient and genotype independent, is therefore desirable. Towards this end, it is noteworthy that strawberry researchers have paid limited attention to somatic embryogenesis. This process has only been described in a few cultivars, and, as far as we know, somatic embryos have not been used in genetic transformation studies. This system has some advantages over adventitious regeneration, such as the higher availability of explants for agroinfection, the possibility of exerting a more controlled selection procedure, or the conversion to rooted plants in a single step. Other authors have proposed an alternative way to achieve this, consisting of the identification

of easily transformed lines that could be used as model genotypes in functional genomics, both in cultivated (Folta *et al*. 2006) and wild strawberry (Oosumi *et al*. 2006; Slovin *et al*. 2009). The usefulness of this strategy still needs to be addressed.

Despite the problem of low transformation efficiency and reproducibility, other aspects of strawberry transformation should be investigated. Few studies have been devoted to analyze the occurrence of somaclonal variation in a population of transgenic strawberries, although it is well known that this process is a source of unintended effects and may influence transgene expression (Bhat and Srinivasan 2002). Environmental risk analysis of transgenic strawberry is also needed for a future field release of these plants. Some authors have indicated that the potential risk of transgenic strawberries is quite low, but this should be demonstrated with deeper studies (Quesada *et al*. 2007). Finally, the development of transformation procedures for wild strawberry other than *F. vesca*, would be beneficial for fundamental studies and also for breeding purposes.

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