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Review

Attempts at grapevine (*Vitis vinifera* L.) breeding through genetic transformation: The main limiting factors

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Summary

Breeding through genetic transformation offers the possibility to add or modify single traits in cultivars without changing desirable characteristics. In grapevine this technology has been scarcely used, taking into account the economic importance of this crop. Up to now, the breeding of grapevine through genetic transformation has been mainly focused on biotic stress resistance, mainly to fungi and viruses. Among the factors that can explain the limited reports of success are the difficulty in regenerating transgenic plants, the availability of only a few characterized genes, and/or the quantitative character of the trait. Another influencing factor is the negative perception of consumers, mainly in Europe. In this review, we discuss the methodology and factors that have limited the success of grapevine transformation, as well as outlining the attempts at breeding grapevine through genetic transformation reported so far. It is expected that the use of transformation, a powerful tool for breeding plants, will increase in grapevine in the coming years as a consequence of the growing knowledge of the function and regulation of grapevine genes and promotors, and of technologies for gene editing.

Introduction

Grapevine (*Vitis vinifera* L.) production had an international value of 44 billion dollars in 2013 (FAOStat 2013) and the global production of grapes was 27.5 million tons. Grapevine was domesticated more than 5000 years ago (Yamamoto *et al*. 2000) and it is considered nowadays the world's most widely-grown fruit crop (Mukherjee *et al*. 2010). Although the genus *Vitis* includes ca. 60 inter-fertile species, *V. vinifera* is the one used most in the global wine industry. *V. lambrusca,* native to North America, also contributes to the development of hybrids for wine and juice production, in certain areas where the climatic conditions may limit *V. vinifera* production (Lee *et al*. 2006). Other species of this genus are employed as grapevine rootstocks due to their resistance to pests (Phylloxera, nematodes), drought tolerance, salt tolerance or tolerance of high pH (Keller 2010). Although there are almost 10,000 cultivars of grapevine (Robinson *et al*. 2012), only a few - including 'Cabernet Sauvignon', 'Merlot', 'Airén', 'Tempranillo', 'Chardonnay', 'Syrah', 'Garnacha Tinta', 'Sauvignon Blanc', 'Trebbiano Toscano', and 'Pinot Noir' - are grown widely (Anderson and Nelgen 2011, Wine Australia - Research, Development and Extension: www.research.wineaustralia.com).

The breeding of scion cultivars has been focused mainly on obtaining resistance to different pathogens. Among the most devastating fungal diseases are powdery mildew, anthracnose, downy mildew, and gray mold rot, caused by *Erysiphe necator*, *Elisinoe ampelina*, *Plasmopara viticola*, and *Botyris cinerea,* respectively (De Francesco 2008, Wilcox 2011). Other important infections are caused by bacteria like *Xylophilus ampelinus*, which can lead to a serious reduction in grapevine health and major harvest losses (Serfontein *et al*. 1997), *Xylella fastidiosa*, which causes Pierce's disease, which can kill the vine in one or two years (Hill and Purcell 1995, Janse and Obradovic 2010), and *Agrobacterium tumefaciens* biovar 3 (*Agrobacterium vitis*), which causes crown gall disease and induces tumors at wounds on the trunks and canes of the grapevine that can necrotize the plant (Burr *et al*. 1998, Ridé *et al*. 2000). Viruses cause other diseases that greatly affect grapevines, especially the Grapevine FanLeaf Virus (GFLV), belonging to the genus *Nepovirus*, which causes the grapevine fanleaf disease. Other breeding goals for grapevine are related to abiotic stress tolerance and quality improvement (Vivier and Pretorius 2002, Gray *et al*. 2014).

Genes conferring resistance to several pathogens have been introgressed into *V. vinifera* cultivars (Alleweldt 1990, Mullins *et al.* 2004). However, the high level of heterozygosity and the long generation cycle in grapevine make breeding by conventional methods difficult (Nakano *et al*. 1994, Franks *et al.* 1998). Therefore, breeding through genetic transformation has been another approach used to incorporate desirable genes into grapevine. This technique offers the possibility of adding single traits to cultivars without, in theory, changing desirable characteristics (Gray *et al*. 2005). The sequencing of the *V. vinifera* genome (Jaillon *et al*. 2007, Velasco *et al*. 2007, Adam-Blondon *et al*. 2011) - which contains over 30,400 genes (Pertea and Salzberg 2010) - has yielded the possibility of obtaining more cisgenic

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plants; that is, those containing genes or regulatory sequences in a sense orientation, which have been isolated from a crossable donor plant. In the work of Schouten *et al*. (2006), Fan *et al*. (2008), Dhekney *et al*. (2011), Dabauza *et al*. (2015), Dai *et al.* (2015), and Dalla-Costa *et al*. (2015), *V. vinifera* genes were introduced mainly by genetic transformation mediated by *Agrobacterium tumefaciens* (updated scientific name *Rhizobium radiobacter*). The availability of genes and emergent technologies, like the CRISPR/Cas system for genome modification (Wang *et al.* 2016) should increase the breeding attempts in grapevine.

Here, we provide an overview of the published work in which attempts at the stable transformation of *V. vinifera* were made, discussing the methodology and factors that have limited the success of grapevine transformation.

Discussion

Despite the fact that *Vitis* sp. is considered to be a natural host for *A. tumefaciens* (Bornhoff *et al*. 2005), T-DNA transfer and its integration into the plant genome by *Agrobacterium* sp. as well as by biolistic methods have been used to incorporate specific genes. To the best of our knowledge, the first attempt to obtain stable transgenic plants in grapevine was reported in 1989 by Baribault *et al*., who used *A. tumefaciens* infection to introduce the neomycin phosphotransferase gene (*nptII*), which confers resistance to kanamycin (Kan), into the cultivar 'Cabernet Sauvignon'. These and other early studies (Baribault *et al*. 1990, Mullins *et al*. 1990, Guellec *et al*. 1990) met with limited success. With respect to biolistics, Hébert *et al*. (1993) reported the first transformation in 'Chancellor', a *Vitis* complex interspecific hybrid, and Scorza *et al*. (1995) were the first group to achieve transformation by a biolistic approach in seedless table grapes. Nowadays, biolistics is the method of choice for studying transient expression for functional analysis. Recent reviews concerning transient expression were provided by Vidal *et al*. (2010) and Jelly *et al*. (2014). Regarding the use of genetic transformation for the breeding of grapevine plants, *Agrobacterium*-mediated transformation has been used mainly (Tab. 1). The combination of both methodologies (biolistics previous to *Agrobacterium* infection) was also tested by Scorza *et al*. (1995 and 1996). One of the advantages of *Agrobacterium*-mediated transformation, with respect to biolistic methods, is the low gene copy number that is obtained in plants regenerated with the former methodology (Li *et al*. 2006, Dutt *et al*. 2008).

Despite the great interest in this technology, it has not been used extensively because of the failure of genetic transformation and/or of the recovery of transgenic plants (Scorza *et al.* 1996, Nookaraju and Agrawal 2012).

Limiting factors - the genotype: Independent of the methodology of gene transfer, the most influential factor regarding the success of transformation is the genotype. Before an attempt to generate transgenic plants is made, an efficient protocol of adventitious regeneration is needed; this will be greatly influenced by the genotype and culture conditions (explant, culture media, etc.). Interspecific and intraspecific variability for regeneration ability were commonly found. Different QTLs have been related to regeneration in different species (Priyono *et al.* 2010, Trujillo-Moya *et al.* 2011, Zhenzhen *et al*. 2015), which manifests the implication of several genes in the regeneration process. On the other hand, each genotype manifests a specific sensitivity to the *Agrobacterium* strain, as well as to the selective agents added to the medium to impede regeneration from non-transgenic cells and to the antibiotics applied to eliminate *Agrobacterium* after transformation (Zhou *et al.* 2014). Differing, genotype-dependent toxicity may also be manifested in biolistic assays according to the type of particle that carries the DNA (Franks *et al*. 1998, Vidal *et al.* 2003).

In *Agrobacterium*-mediated transformation, success will be influenced also by the ability of the cells of each genotype to be transformed, the concentration of bacteria, the time of co-culture, and even the genetic constructions used. In biolistic transformation, the size of particles coated with DNA, the helium pressure, the gap distance, the vacuum, and the distance from the carrier to the sample also may be factors that will influence the transgenic efficiency. Both gold (Scorza *et al*. 1995 and 1996, Franks *et al.* 1998, Vidal *et al*. 2003 and 2006) and tungsten (Hébert *et al*. 1993, Vidal *et al*. 2003) particles, of 0.6, 0.75, 1, and 1.6 microns in size, have been used to carry the DNA in grapevine.

Adventitious regeneration: Adventitious regeneration may occur via organogenesis or embryogenesis. In *Vitis* sp. adventitious regeneration is mainly achieved through somatic embryogenesis and much research has been carried out using, as starting explants: leaves (Martinelli *et al*. 1993, Nakano *et al*. 1994, Scorza *et al*. 1995 and 1996, Das *et al*. 2002, Bornhoff *et al*. 2005, Li *et al*. 2006, Dutt *et al*. 2008, Nirala *et al*. 2010, Dhekney *et al*. 2011, Nookaraju and Agrawal 2012, Li *et al.* 2015), anthers (Franks *et al*. 1998, Iocco e*t al*. 2001, Nakajima and Matsuda 2003, Vidal *et al*. 2003 and 2006, Perrin *et al*. 2004, Agüero *et al*. 2005, Gambino *et al*. 2005, Fan *et al*. 2008, López-Pérez *et al*. 2008, Rosenfield *et al*. 2010, Wang *et al*. 2005, Dai *et al.* 2015), ovaries (Yamamoto *et al*. 2000, Vidal *et al*. 2003 and 2006, Gambino *et al*. 2005, Rosenfield *et al*. 2010, Dai *et al*. 2015), whole flowers (Dai *et al.* 2015), mature seeds (Peiró *et al*. 2015), stigmas and styles (Morgana *et al.* 2004, Carimi *et al.* 2005), petioles (Robacker 1993), tendrils (Salunkhe *et al.* 1997), nodal sections (Maillot *et al.* 2006), and protoplasts (Reustle *et al*. 1995, Zhu *et al*. 1997, Xu *et al*. 2007). Although regeneration has been obtained in several cultivars, the germination of aberrant embryos that may limit regeneration or decrease the real percentage of regenerated grapevine plants is a common occurrence. Embryos without cotyledons, with different numbers of cotyledons (mono-, di-, and poly-cotyledonary), or with fused cotyledons, and trumpet-shaped or cauliflower-like cotyledons were described in different works (Goebel-Tourand *et al*. 1993, Martinelli *et al*. 2001, Bornhoff *et al*. 2005, Li *et al*. 2006, López-Pérez *et al*. 2006, Bharathy and Agrawal 2008, Martinelli and Gribaudo 2009 or Peiró *et al.* 2015). The majority of these abnormal embryos do not develop into normal plants. For grapevine transformation three types of embryogenic tissue are commonly used: somatic embryos from leaves (Scorza *et al*. 1995 and 1996, Li *et al*.

Table 1

Reports of stable transformation in grapevine: methodology and regeneration of transgenic plants

Cultivar	Method	Selection	Number of regenerated plants	Analyses for Integration	Objective	Reference
Cabernet Sauvignon	*A. tumefaciens*[1]	Kanamycin (10 in liquid culture) Kanamycin (up to 50 in plates)	No plants obtained	-	Protocol development	Baribault *et al.* 1989
Cabernet Sauvignon, Chardonnay, Grenache, Riesling	*A. tumefaciens*	Kanamycin (100)	Data not shown	7 (not indicated)	Protocol development	Huang and Mullins 1989
Sultana, Cabernet Sauvignon	*A. tumefaciens*	Kanamycin (10 to 25)	No plants obtained	-	Protocol development	Baribault *et al.* 1990
Grenache	*A. rhizogenes*	Kanamycin (25)	No plants obtained	-	Protocol development	Guellec *et al.* 1990
Cabernet Sauvignon, Chardonnay	*A. tumefaciens*	Kanamycin (0 to 25)	No plants obtained	-	Protocol development	Mullins *et al.* 1990
Thompson Seedless, French Colombard	*A. tumefaciens*	Kanamycin (3 to 7)	No plants obtained	-	Protocol development	Colby *et al.* 1991
Koshusanjaku	*A. rhizogenes*	Kanamycin (50)	12	12 (Southern Blot+)	Protocol development	Nakano *et al.* 1994
3 seedless grape selections	Biolistic + *A. tumefaciens*	Kanamycin (first 6 w 20 and next 6 w 40)	14 (results of the three cultivars shown together)	14 (PCR+)	Protocol development	Scorza *et al.* 1995
Chardonnay	*A. tumefaciens*	Paramomycin (5 to 20)	50	46 analyzed/30 (GUS+)	Protocol development	Mauro *et al.* 1995
Superior Seedless	*A. tumefaciens*	Kanamycin (50 to 500) Hygromycin (15 to 25) Basta (1 to 10)	Hygromycin (25) 86 Basta (10) 67	60 (PCR+ and Southern Blot+) 42 (PCR+)	Protocol development	Perl *et al.* 1996
Thompson Seedless	Biolistic + *A. tumefaciens*	Kanamycin (40, 6 w after co-cultivation)	13	13 (PCR+ and Southern Blot+)	Protocol development	Scorza *et al.* 1996
Russalka	*A. tumefaciens*	Kanamycin (100 directly after two months of co-culture or a monthly stepwise of 12.2, 25, 50)	Data not shown	Data not shown	Protocol development	Gölles *et al.* 1997
Sultana	Biolistic + *A. tumefaciens*	Kanamycin 1 (30, following co-cultivation and 8-10 w later, 100 or stayed at 50). Kanamycin 2 (50, 3 w after co-cultivation and, 2.5-3 w later, 100) Higromycin (25)	63 12 12	4 (GUS+ and Southern Blot+) 11 (") 11 (")	Protocol development	Franks *et al.* 1998
Russalka	*A. tumefaciens*	Kanamycin (75 or 100)	Data not shown	140 (PCR+)	Protocol development	Gölles *et al.* 1998
Dornfelder, Müller-Thurgau, Riesling	*A. tumefaciens*	Kanamycin (100 and, 4 w later, 50)	Data not shown	Data not shown	Protocol development	Harst *et al.* 2000a
Dornfelder, Riesling, Müller-Thurgau	*A. tumefaciens*	Kanamycin (100 solid medium, 100 and, after 4 w, 50 in liquid medium)	Data not shown	Data not shown	Protocol development	Harst *et al.* 2000b
Neo Muscat	*A. tumefaciens*	Kanamycin (50)	More than 20	9 analyzed/7 (PCR+) 5 (Southern Plot+)	Disease resistance	Yamamoto *et al.* 2000
Cabernet Sauvignon, Shiraz, Chardonnay, Riesling, Sauvignon Blanc, Chenin Blanc, Muscat Gordo Blanco	*A. tumefaciens*	Kanamycin (100)	Cardonnay 52 Cabernet Sauvignon 136 Sauvignon blanc 23 Chenin Blanc 57 Muscat Gordo Blanco 9 Shiraz 161 Riesling 19 Semillon 0 Pinot noir 0	7 (Southern Blot+) 31 (") 5 (") 19 (") 1 (") 45 (") 2 (") - -	Protocol development and comparison of transformation efficiency of different varieties	Iocco *et al.* 2001

Tab. 1, continued

Cultivar	Method	Selection	Number of regenerated plants	Analyses for Integration	Objective	Reference
Russalka	*A. tumefaciens*	Kanamycin (100, 2 months after co-cultivation, or 25 after co-cultivation with a monthly stepwise)	15	2 (PCR+)	Protocol development	Gutoranov *et al.* 2001
Chardonnnay, Shiraz, Danuta, Portan	*A. tumefaciens*	Kanamycin (100 to 150 in 3-4 w)	Portan 20 Shiraz 8 Danuta 0 Chardonnay 0	16 (PCR+ and Southern Blot+) 4 (") - -	Protocol development	Torregrosa *et al.* 2002
Silcora, Thompson Seedless	*A. tumefaciens*	Kanamycin (25 the first 30 d, then 50 the next 30 d, and finally 75 the last 30 d)	50	Silcora 10 tested/8 (Southern Blot+). 2 tested/2 (RT-PCR+) Thompson Seedless 5 tested/3 (Southern Blot+). 1 tested/1 (RT-PCR+)	Protocol development	Mezetti *et al.* 2002
Pusa Seedless, Beauty Seedless, Perlett, Nashik	*A. tumefaciens*	Kanamycin (20)	Data not shown	Data not shown	Protocol development	Das *et al.* 2002
Chardonnay	Biolistic	Kanamycin (10 and, 4 w later, 15)	260	148 analyzed/71 (PCR+) 83 (DBH+)	Protocol development	Vidal *et al.* 2003
Thompson Seedless, Chardonnay	*A. tumefaciens*	Kanamycin (100)	Thompson Seedless 36 Chardonnay 15	32 (PCR+) 11 (")	Disease resistance	Agüero *et al.* 2005
Seyval blanc	*A. tumefaciens*	Kanamycin (100)	Data not shown	Data not shown	Disease resistance	Bornhoff *et al.* 2005
Nebbiolo, Blaufränkisch, Lumassina	*A. tumefaciens*	Kanamycin (50, one month after co-cultivation, and increasing to 100 since the second month of culture)	Nebbiolo 40 Lumassina 1 Blaufränkisch 2	33 (PCR+ and RT-PCR) 40 (Southern Blot+) (22 ELISA+) 1 (") 1 (") (ELISA not tested) 2 (") 2 (") (ELISA not tested)	Protocoll development with GFLV resistance genes	Gambino *et al.* 2005
Red Globe	*A. tumefaciens*	Paramomycin (stepwise 5 to 20) Kanamycin (between 40 and 100)	Data not shown	Data not shown	Protocol development	Wang *et al.* 2005
Thompson Seedless	*A. tumefaciens*	Kanamycin (25, 50, 75, 100 or 150)	795	Data not shown	Protocol development	Li *et al.* 2006
Chardonnay, Thompson Seedless	*A. tumefaciens*	Kanamycin (100)	Data not shown	Thompson Seedless 191 GUS+ (Fluorescence) 180 PGIP or GFP+ (Radial diffusion assay and fluorescence) 173 GUS and PGIP or GFP+ Chardonnay 41 GUS+ (Fluorescence) 39 PGIP or GFP+ (Radial diffusion assay and fluorescence) 37 GUS and PGIP or GFP+	Protocol development	Agüero *et al.* 2006
Chardonnay	Biolistic	Kanamycin (10 and, 4 w later, 15)	19	19 (PCR+) 17 (Southern Blot+) 8 RT-PCR+) for mag2 9 (") for MSI99	Disease resistance	Vidal *et al.* 2006
Thompson Seedless	*A. tumefaciens*	Kanamycin (16)	Data not shown	18 (PCR+) 4 randomly selected (Southern Blot+)	Protocol development	Dutt *et al.* 2007
Thompson Seedless	*A. tumefaciens*	Kanamycin (100 and 30 d later, 70)	25	18 (PCR+)	To produce transgenic plants without marker genes	Dutt *et al.* 2008

Tab. 1, continued

Cultivar	Method	Selection	Number of regenerated plants	Analyses for Integration	Objective	Reference
Thompson Seedless	*A. tumefaciens*	Hygromycin (3, 6, 9, 12 or a stepwise selection with the same concentration)	27	19 (PCR+) 9 analyzed/5 (Southern Blot+)	Protocol development and to find the optimal concentration of hygromycin	Fan *et al.* 2008
Sugraone, Crimson Seedless	*A. tumefaciens*	Kanamycin (5, 10, 20, 30, 40, 50)	Crimson Seedless 28 (kan 20) Sugraone 22 (kan 50)	26 (PCR+) 21 (")	Protocol development and to find the optimal concentration of Kan	López-Pérez *et al.* 2008
Thompson Seedless, Merlot, Shiraz	*A. tumefaciens*	Kanamycin (20) Kanamycin (50)	Data not shown	Thomson Seedless 17 analyzed/17 (Southern Blot+)	Protocol development	Li *et al.* 2008
Centennial Seedless	*A. tumefaciens*	Kanamycin (10)	45	39 (PCR+) 4 analyzed/4 (Southern Blot+)	Cold resistance	Jin *et al.* 2009
Pusa Seedless	*A. tumefaciens*	Hygromycin (10 the first 20 d, then 15 or 20) Hygromycin (25)	Data not shown	7 analyzed/7 (PCR+) for hpt 4 (PCR+) for pGL2 4 analyzed/4 (Southern Blot+) 5 analyzed/4 (RT-PCR+) 4 analyzed/4 (Western Blot+)	Disease resistance	Nirala *et al.* 2010
Chardonnay	Biolistic	Data not shown	Data not shown	28 (PCR+)	Disease resistance	Rosenfield *et al.* 2010
Thompson Seedless	*A. tumefaciens*	Kanamycin (100)	71	2 (ELISA+)	Disease resistance	Dhekney *et al.* 2011
Albariño	*A. tumefaciens*	Kanamycin (50)	52	7 (PCR+) 5 analyzed/4 (Southern Blot+)	Disease resistance	Gago *et al.* 2011
Crimson Seedless	*A. tumefaciens*	Hygromycin (5, 2 w after co-culture and, after 2 w more, 10)	Data not shown	Data not shown	Disease resistance	Nookaraju and Agrawal 2012
Thompson Seedless	*A. tumefaciens*	Kanamycin (25) Kanamycin (50) Kanamycin (75) Kanamycin (100) Kanamycin (150)	351	16 (PCR+) 17 (") 32 (") 28 (") 18 (")	Protocol development and disease resistance	Zhou *et al.* 2014
Sugraone	*A. tumefaciens*	Kanamycin (50 after 10 d of the co-culture)	26	2 analyzed/2 (Southern Blot+) 2 analyzed/2 (RT-PCR+)	Disease resistance	Dabauza *et al.* 2015
Chardonnay	*A. tumefaciens*	Hygromycin (10)	69	32 (Southern Blot+)	Protocol development and disease resistance	Dai *et al.* 2015
Thompson Seedless	*A. tumefaciens*	Kanamycin (not shown)	870	Data not shown. Analyses of random plants	Disease resistance	Li *et al.* 2015
Chardonnay, Shiraz, Cabernet Sauvignon, Malian, Shalistin, Pinot Noir	*A. tumefaciens*	Kanamycin (100)	Data not shown	Data not shown	Study of anthocyanins in transgenic grapevines	Rinaldo *et al.* 2015
Brachetto	*A. tumefaciens*	Kanamycin (150 after 4 w)	12	6 analyzed/6 (PCR+)	Cisgenesis protocol	Dalla-Costa *et al.* 2016

[1] *A. tumefaciens* classified now as *Rhizobium radiobacter*.

2006, Dutt *et al*. 2008, Dhekney *et al*. 2011, Nookaraju and Agrawal 2012, Li *et al*. 2015), anthers (Franks *et al*. 1998), or ovules (Yamamoto *et al.* 2000)·; embryogenic calli from leaves (Nakano *et al*. 1994, Nirala *et al*. 2010), anthers (Franks *et al*. 1998, Iocco *et al*. 2001, Agüero *et al*. 2005, Gambino *et al*. 2005, Fan *et al*. 2008, López-Pérez *et al*. 2008, Dai *et al*. 2015), ovaries (Gambino *et al*. 2005, Dai *et al*. 2015), or whole flowers (Dai *et al*. 2015); and cell suspensions from anthers (Franks *et al*. 1998, Vidal *et al.* 2003 and 2006, Wang *et al*. 2005, Rosenfield *et al*. 2010) or ovaries (Vidal *et al*. 2003 and 2006, Rosenfield *et al*. 2010). Leaf disks, microshoots, or meristematic cell clusters were also used by Bornhoff *et al*. (2005), Gago *et al*. (2011), and Mezetti *et al*. (2002a), respectively.

About half of the reports in Table 1 used 'Chardonnay' and 'Thompson Seedless' for grape transformation and great variability was reported with respect to the number of plants regenerated in selective conditions. In Iocco *et al*. (2001), the effect of genotype is clear. With similar transformation conditions, the number of plants regenerating under selective conditions (Kan applied 3 weeks after co-culture) greatly differed among cultivars: 161 plants of 'Shiraz', 136 'Cabernet Sauvignon', 57 'Chenin Blanc', 52 'Chardonnay', 23 'Sauvignon Blanc', 19 'Riesling', and nine 'Muscat Gordo Blanco' were obtained, but no 'Semillon' or 'Pinot Noir'.

Effects of the Agrobacterium strain and selective agent on regeneration: In different studies, it is reported that the competence of *Agrobacterium* mediated transformation is cultivar dependent (Iocco *et al*. 2001, Dutt *et al*. 2011). In addition, after *Agrobacterium* inoculation, necrosis and tissue browning - as a result of an oxidative burst caused by reactive oxygen species - may appear in some genotypes (Perl *et al*. 1996, Gustavo *et al*. 1998, Li *et al.* 2006, Zhou *et al*. 2014), affecting regeneration. These facts, associated with the sensitivity to the selective agents (which depend on the gene inserted for selection), together with the putative sensitivity to the antibiotics used for elimination of *Agrobacterium* (that will differ depending on the virulence of the bacteria), explain the difficulty in obtaining efficient protocols for grapevine transformation.

Among the disarmed *A. tumefaciens* strains LBA4404 (Hoekma *et al.* 1983), GV2206 (Ryder *et al.* 1985), EHA101 and EHA105 (Hood *et al.* 1993), GV3101 (Holsters *et al.* 1980), and AGL1 (Lazo *et al.* 1991) (all derive from C58, with the exception of LBA4404 - that derives from Ach5), EHA105 is the one employed most in grapevine transformation (Scorza *et al*. 1996, Franks *et al*. 1998, Iocco *et al*. 2001, Wang *et al*. 2005, Dhekney *et al*. 2007 and 2011, Dutt *et al*. 2008, Dabauza *et al*. 2015, Li *et al*. 2015). Despite the fact that regeneration was obtained by Nakano *et al*. (1994) and Franks *et al*. (2006) using *Agrobacterium rhizogenes*, no more reports were found in grapevine.

In order to restrict regeneration to cells which have incorporated the transgene, the *nptII* gene (Tab. 1) - that confers resistance to kanamycin (Kan) and other antibiotics like paramomycin, neomycin, and G418 - has been the one used most commonly in grapevine transformation (Nakano *et al*. 1994, Scorza *et al*. 1995 and 1996, Franks *et al*. 1998, Yamamoto *et al*. 2000, Iocco *et al*. 2001, Vidal *et al.* 2003 and 2006, Agüero *et al*. 2005, Bornhoff *et al*. 2005, Gambino *et al*. 2005, Wang *et al.* 2005, Li *et al*. 2006, Dutt *et al*. 2008, López-Pérez *et al*. 2008, Jin *et al*. 2009, Dhekney *et al*. 2011, Gago *et al*. 2011, Dabauza *et al*. 2015, Li *et al.* 2015). However, high sensitivity of grapevine tissues to Kan was reported in Gray and Meredith (1992) and different authors mention that it is really difficult in grapevine to balance the concentration of Kan that is adequate for selection which allows development of embryos and shoots (Gray and Meredith 1992, Torregrosa *et al*. 2000, Saporta *et al.* 2014). This explains the great differences with respect to the Kan concentration used for selection and the time of application of the selective agent (Tab. 1). In addition to the genotype, the kind of explant used (callus, suspension cultures…) for transformation will lead to differences in sensitivity (Zhou *et al*. 2014). Kanamycin is used in a range from 10 to 100 $mg \cdot L^{-1}$ (Tab. 1). Regarding the application of the selective agent, Franks *et al*. (1998) compared three strategies, achieving better results when they applied low selection (2 $mg \cdot L^{-1}$ Kan) at the beginning, moderate selection (50 $mg \cdot L^{-1}$ Kan) three weeks after co-cultivation, and 100 $mg \cdot L^{-1}$ Kan thereafter. Good regeneration was also obtained by Iocco *et al*. (2001), who added 100 $mg \cdot L^{-1}$ Kan after three weeks of co-culture. Although they found a high number of escapes, some transgenics were selected in all the cultivars showing regeneration. In Wang *et al.* (2005), Kan and paromomycin were compared as selective agents for 'Red Globe', with better results for transformation efficiency and embryo development being obtained with the latter at 20 $mg \cdot L^{-1}$. However, the use of this antibiotic is not common in transgenic work.

The *hptI* gene, that encodes hygromycin (Hyg) phosphotransferase I, is the second selective gene used, to select transformed grapevine cells in Hyg-containing media (Franks *et al.* 1998, Fan *et al.* 2008, Nirala *et al.* 2010, Nookaraju and Agrawal 2012, Dai *et al.* 2015). The Hyg concentrations employed ranged from 3 to 25 $mg \cdot L^{-1}$ (Franks *et al*. 1998, Fan *et al*. 2008, Nirala *et al*. 2010, Nookaraju and Agrawal 2012, Dai *et al*. 2015). Whereas Franks *et al.* (1998) obtained a good selection efficiency using 25 $mg \cdot L^{-1}$ Hyg (11 of 12 regenerated plants of cv. 'Sultana' were confirmed as transgenic), Fan *et al.* (2008) found toxicity during direct selection with 12 $mg \cdot L^{-1}$ Hyg in 'Thompson Seedless'. A stepwise selection with 3, 6, 9, and finally 12 $mg \cdot L^{-1}$ gave a high transformation efficiency (72 %). In Nirala *et al*. (2010) a stepwise selection was also performed, for the cultivar 'Pusa Seedless'; however, in this work the first selection was made with 10 $mg \cdot L^{-1}$ Hyg. This concentration was also used for selection in 'Chardonnay' by Dai *et al*. (2015), who recovered normal and abnormal embryos (deformed leaves, vitrification, no development of roots). In Saporta *et al*. (2014) a comparison of Kan and Hyg was performed for cellular suspensions of cultivar 'Albariño', yielding an optimal selection pressure of 20-40 $mg \cdot L^{-1}$ and 5-10 $mg \cdot L^{-1}$ for the former and the latter antibiotic, respectively.

The genes *bar* (Perl *et al.* 1996) and *pmI* (Reustle *et al*. 2003, Kieffer *et al*. 2004, Jardak-Jamoussi *et al*. 2008) - that encode, respectively, a phosphinothricin acetyl transferase and a phosphomannose isomerase - have also been used as

selective agents in grapevine transformation, with success.

Even when using similar selective conditions (100 mg·L^{-1} Kan, 3 weeks after co-culture) and explant type (embryogenic cultures from immature anthers), different regeneration was obtained for a specific cultivar. For instance, in 'Shiraz', Iocco *et al.* (2001) obtained 161 plants whereas only eight were reported in Torregrosa *et al.* (2002). In the former study 28 % integration was found, with 25 % in the latter. In the cultivar 'Chardonnay', Torregrosa *et al*. (2002) could not regenerate plants; however, Iocco *et al*. (2001) obtained 52 plants (13 % confirmed as transgenic). Probably, these great differences are due to other factors that influence the protocol.

In some studies confirmation of integration and/or expression was not performed, the authors assuming that the plants regenerated under selective conditions were transgenic (Iocco *et al*. 2001, Li *et al*. 2006, Wang *et al*. 2005, Bornhoff *et al*. 2005). From the results in Tab. 1 we can conclude that, despite the fact that in some cases 100 % of the regenerated plants were confirmed as transgenic, in the majority of the studies escapes were regenerated.

The European Food Safety Authority (EFSA 2009) recognizes *nptII* as a safety gene; however, different strategies have been developed and are available to recover *nptII*-free plants after selection. In grapevine a co-transformation system was reported by Dutt *et al*. (2008), who were trying to produce transgenic grapevines free of marker genes. They used an *Agrobacterium* strain which contained a binary plasmid with an *egfp* gene of interest for positive selection and, for negative selection, the cytosine deaminase (*codA*) gene, the two genes linked by a bi-directional dual promoter complex. Dalla-Costa *et al*. (2009 and 2010) employed the XVE-Cre/LoxP system to induce removal of the *nptII* gene, induced by 17-β-estradiol. Calli, leaves, and roots from the Italian cultivar 'Brachetto' were used and transgenic plants without the *nptII* gene were obtained, suggesting that the use of XVE-Cre/LoxP could be a good method for elimination of selectable gene markers.

Attempts at grapevine (*Vitis vinifera* L.) breeding through genetic transformation.

Breeding for resistance to fungi and bacteria: As a consequence of pathogen attack, a number of pathogenesis-related (PR) proteins are produced in grapevine (Jacobs *et al*. 1999); among them the glucanases and chitinases are the most common. Besides PR proteins, the accumulation of phytoalexins - such as stilbenes - is the other major defense mechanism frequently observed in grapevine (Ferreira *et al*. 2004). These kinds of antifungal related genes have been the ones used most commonly for grapevine breeding through genetic transformation (Tab. 2). For instance, the rice chitinases (*RCC2* and *Chil1*) were introduced by Yamamoto *et al*. (2000) and Nirala *et al*. (2010) into 'Neo Muscat' and 'Pusa Seedless', respectively. In these works, the transformants had higher levels of chitinase activity and tended to have smaller lesions when they were affected by anthracnose and powdery mildew, with respect to the control plants. Kikkert *et al*. (2000) also transformed 'Merlot' and 'Chardonnay' with an endochitinase gene from *Trichoderma harzianum* (*ThEn-42*), obtaining similar results: the transformants had 10 to 100-fold higher chitinase activity relative to the controls but showed low levels of resistance to powdery mildew and a reduced incidence and severity of symptoms for Botrytis bunch rot, in both greenhouse and field evaluations (Kikkert *et al*. 2009). Other biocontrol agents derived from *Trichoderma* spp. - like two endochitinase (*ech42* and *ech33*) genes and one *N*-acetyl-b-D-hexosaminidase (*nag70*) gene - were introduced into 'Thompson Seedless' by Rubio *et al*. (2015), who obtained several lines with consistent resistance. Recently, Dai *et al.* (2016) used a *Vitis pseudoreticulata* PR gene (*VpPR4-1*) to transform the cv. 'Red Globe'. Six plants inoculated with powdery mildew showed resistance. The use of other chitinases, in combination with ribosome inactivation proteins (Harst *et al*. 2000a, Bornhoff *et al*. 2005) or beta 1-3 glucanases (Harst *et al*. 2000a, Nookaraju and Agrawal 2012), has also yielded results similar to those of the above mentioned works. Other plant PR proteins are the thaumatin-like proteins (TLPs), which are grouped into the PR-5 family. A *V. vinifera* gene (*Vvtl-1*) encoding a TLP was introduced into 'Thompson Seedless' and enhanced resistance to foliar fungal diseases and lowered the incidence of sour rot in berries (Dhekney *et al*. 2011). Also, genes encoding stilbene synthase - a key enzyme that produces trans-resveratrol, the major phytoalexin in grape - were introduced into 'Chardonnay' (Dai *et al*. 2015), 'Sugraone' (Dabauza *et al*. 2015), and 'Thompson Seedless' (Fan *et al*. 2008). Reduced numbers of powdery mildew conidia and smaller lesions after infection with *B. cinerea* were reported in the first and second studies cited.

Other strategies to achieve resistance to fungi were the insertion of a polygalacturonase inhibiting protein (Agüero *et al.* 2005) and the introduction of antimicrobial genes (lytic peptides) like Shiva-1, *mag2*, *MSI99*, and *PGL* (Scorza *et al.* 1996, Vidal *et al*. 2003 and 2006, Rosenfield *et al*. 2010) (Tab. 2). Whereas the low transformation efficiency impeded the evaluation of resistance by Scorza *et al*. (1996), a delay in the *A. vitis* infection or in the expansion of lesions in transgenic lines, with respect to the control, was reported by Kikkert *et al*. (2009) in plants with *mag-2* and *MSI-99* genes. More recently, Dandekar *et al.* (2012) introduced a PGIP signal peptide with a cecropin derived lytic domain and Li *et al*. (2015) introduced the gene *LIMA-A* (that also encodes a lytic peptide derivative of *MsrA1*), in order to confront Pierce's disease. Although the plants showed resistance in the greenhouse, no durable resistance was obtained in the field - where all plants died before the seventh year of cultivation.

Transgenic grapevines for resistance to viruses and other pathogens: The first authors to obtain virus resistant grape plants through genetic transformation, like in other species, used pathogen-derived resistance: concretely, the insertion of virus coat proteins (CP) (Tab. 3). Mauro *et al*. (1995) and Scorza *et al*. (1996) reported the transformation of cultivars 'Chardonnay' and 'Thompson Seedless' with a CP of GFLV and the Tomato Ringspot Virus CP (TomRSV-CP). A similar strategy was used in different studies which attempted to achieve GFLV resistance (Gölles *et al*. 1997, Tsvetkov *et al*. 2000, Gu-

Table 2

Transgenic reports focused on the incorporation of genes related with fungus and bacterial resistance in grapevine

Cultivar	Gene(s)/Protein(s)	Goal	References
Thompson Seedless	Shiva-1 (lytic peptide gene)	Bacterial resistance	Scorza *et al.* 1996
Chardonnay, Chancelor and Merlot	n.d. Chitinase (Trichoderma endochitinase)	Powdery mildew resistance	Kikkert *et al*. 1997
Riesling and Dornfelder	n.d. Chitinase and n.d. glucanase n.d. Chitinase and n.d. Ribosome inactivation protein (RIP)	Disease resistance	Harst *et al.* 2000
Merlot and Chardonnay	*ThEn-42 (endochitinase gene from Trichoderma harziaru)*	Powdery mildew and Botrytis buch rot resistance	Kikkert *et al*. 2000 & 2009
Neo Muscat	*RCC2* (Rice Chitinase)	Fungal resistance to powdery mildew	Yamamoto *et al*. 2000
Chardonnay	*mag2* (Lytic peptide) *MSI99 (*Sintetic lytic peptide) PGL (Peptidyl-glycine-leucine)	Fungal resistance	Vidal *et al.* 2003 & 2006
Thompson Seedless and Chardonnay	*pPgip* (Pear Polygalacturonase-inhibiting protein gene)	Enhance resistance to Botrytis	Agüero *et al*. 2005
Seyval blanc	n.d. Chitinase and ribosome inactivation protein (RIP from *Hordeum vulgare*)	Antifungal proteins for resistance for Uncinuka necator and *Plasmopara viticola*	Bornhoff *et al.* 2005
Thompson Seedless	*STS* (Stilbene syntase from *Vitis reticulata*)	Phytoalexin for fungal resistance	Fan *et al*. 2008
Pusa Seedless	*Chil1* (Rice Chitinase)	Fungal resistance to powdery mildew	Nirala *et al.* 2010
Chardonnay	*mag2* and *PGL* (Lytic peptide and peptidil-glycine- leucine respesctively)	Fungal resistance to powdery mildew and crown gall.	Rosenfield *et al*. 2010
Thompson Seedless	*Vvtl-1 (Vitis vinifera thaumatin-like protein)*	Fungal resistance for powdery mildew and black rot. Also resistance to sour-bunch rot (bacteria)	Dhekney *et al.* 2011
Thompson Seedless	PGIP signal peptide with a cecropin derived lytic domain	Pierce's disease resistance	Dandekar *et al.* 2012
Crimson Seedless	*Chi1* (Chitinase from scab-infected Sumai-3 wheat) *β-1,3-glucanase* (from same wheat)	Anti-fungal genes for increased tolerance to downy mildew fungus	Nookaraju and Agrawal 2012
Sugraone	*VstI* (Grapevine stilbene syntase)	Enhanced fungal resistance to grey mould (*B. cinerea*)	Dabauza *et al*. 2015
Chardonnay	*VpSTS* (*Vitis pseudoreticulata* stylbene sintase)	Powdery mildew resistance	Dai *et al*. 2015
Thompson Seedless	LIMA-A (Sintetic gene encoding lythic peptide)	Improve Pierce's disease resistance	Li *et al*. 2015
Thompson Seedless	ech42 (Endochitinase) ech33 (Endochitinase) nag70 (N-acetyl-b-Dhexosaminidase gene)	Increase resistance to Botrytis cinerea and Erysiphe necátor	Rubio *et al.* 2015
Red Glove	*VpPR4-1* (Pathogenesis-related protein from *Vitis pseudoreticulata*)	Improve powdery mildew resistance	Dai *et al.* 2016

toranov *et al*. 2001 and Gambino *et al*. 2005) or resistance to ArMV and Grapevine viruses A and B (GVA, GVB) (Gölles *et al*. 1998). Movement virus proteins were also used by Marinelli *et al*. (1998) to achieve resistance to GVA and GVB. Resistance was not reported in these works. More recently, the RNA interference strategy was utilized for stable grapevine transformation, using inverted repeats (Jardak-Jamousssi *et al*. 2008). In this case, a low number of transgenic lines of grapevine were obtained and evaluation of GFLV resistance was not reported.

Important pests of grapevine are Phylloxera (*Daktulosphaira vitifoliae*) and Root knot nematodes (RKN). Despite the problems caused by the former, that are solved by the use of resistant rootstocks, Franks *et al*. (2006) introduced, by genetic transformation, three sequences of Sorghum in order to produce a cyanogenic glycoside that is involved in plant defense mechanisms. However, these transformed plants did not show evidence of greater protection - probably because, after infestation, the accumulation of the metabolite was low. With respect to RKN, yield problems have increased since the withdrawal of methyl bromide. These pests, in addition to reducing yield because their galls limit nutrient acquisition, are virus transmitters. For instance, the dagger nematode (*Xiphinema index*) transmits GFLV, one of the most severe virus diseases of grapevines worldwide. In 'Chardonnay', Yang *et al*. (2013) introduced two hairpin-based silencing constructs, containing two stem sequences of the *16D10* gene, and transformed hairy roots

Table 3

Transgenic reports focused on the incorporation into grapevine of genes related with resistance to viruses and other pathogens

Cultivar	Gene(s)/Protein(s)	Goal	References
Chardonnay	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	Grapevine FanLeaf Virus resistance	Mauro *et al*. 1995
Thompson Seedless	TomRSV-CP (Tomato RingSpot Virus Coat Protein) Shiva-1 (lytic peptide gene)	Virus and bacterial resistance	Scorza *et al.* 1996
Rusalka	GFLV CP (Grapevine FanLeaf Virus Coat Protein) ArMV CP (Arabis Mosaic Virus Coat Protein) GVA CP (Grapevine Virus A Coat Protein) GVB CP (Grapevine Virus B Coat Protein)	Resistance to Grapevine FanLeaf Vrius, Arabis Mosaic Virus, Grapevine Virus A and B	Gölles *et al*. 1997 & 1998
Superior Seedless	MP (Movement Protein)	Grapevine Virus A and B resistance	Martinelli *et al.* 1998
Rusalka	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	Grapevine FanLeaf Virus resistance	Tsvetkov *et al.* 2000
Rusalka	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	Grapevine FanLeaf Virus resistance	Gutoranov *et al*. 2001
Nebbiolo, Blaufränkisch and Lumassina	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	To obtain resistance to Grapevine FanLeaf virus	Gambino *et al*. 2005
Sultana	*CYP79A* and *Cyp71E1* (Encoding cytochrome p450 from *Sorghum*) *sbHMNGT* (UDPG glucosyltransferase-encoding from *Sorghum*)	Phylloxera resistance	Franks *et al.* 2006
Arich Dressé	IR MPc GFLV (Inverted Repeat Silencing Movement Protein from GFLV)	Grapevine FanLeaf Virus resistance	Jardak-Jamoussi *et al*. 2009
Chardonnay	*pART27-42* (RNA interference silencing a conserved root-knot nematode effector gene *16D10*) *pART27-271*	Root-knot nematodes resistance	Yang *et al*. 2013

to test their small interfering RNA (siRNA) production and efficacy of suppression of nematode infection, with promising results. They obtained four lines and better nematode resistance (fewer eggs per root) was observed, with respect to the control.

Cold tolerance, yield, and grape quality: Other reported attempts at enhancing cold tolerance and grape quality in grapevine through genetic transformation are shown in Tab. 4.

Plant stress responses take place through complex and interacting pathways, which indicate the difficulty for breeding with both traditional and biotechnological techniques. However, in grapevine different attempts have been made to achieve cold tolerance. The expression of an Fe-superoxide dismutase or the *VvAdh2* gene of *V. vinifera* - that encodes an alcohol dehydrogenase - was reported by Rojas *et al*. (1997) and Tesnière *et al*. (2006), respectively. In the first of these works a lower sucrose content, a higher degree of polymerization of proanthocyanidins, and an increase in volatile compounds, especially for carotenoid- and shikimate-derived volatiles, were obtained in transgenic plants. On the other hand, Jin *et al*. (2009) and Gutoranov *et al*. (2001), respectively, transformed 'Centennial Seedless' and 'Rusalka' grapevines with genes encoding different

Table 4

Transgenic reports focused on different breeding objectives in grapevine, with the aim of increasing yield by increasing the number of berries or the tolerance of abiotic stresses, particularly low temperatures

Cultivar	Gene(s)/Protein(s)	Goal	References
Cabernet Franc	Fe-superoxide dismutase	Freezing tolerance	Rojas *et al*. 1997
Rusalka	Arf 11 (Antifreeze protein) Arf 62 (Antifreeze protein) Arf 75 (Antifreeze protein) B5 (Antifreeze protein)	Cold resistance	Gutoranov *et al*. 2001
Vitis vinifera (seedless cultivar)	PPOa (Polyphenol oxidase antisense) UFGT (UDP:flavonoid 3-*O*-glucosyltransferase)	Reduction of PPO levels (browning of damaged plant tissues), berry color	Thomas and Scott 2001
Thompson Seedless and Silcora	*DefH9-iaaM* (Protein that increases IAA formation)	Increased number of flowers and berries	Mezzetti *et al*. 2002 and Constantini *et al*. 2007
Portan	*VvAdh2* (*Vitis vinifera* alcohol dehydrogenase)	Abiotic stress resistance	Tesnière *et al.* 2006
Centennial Seedless	*AtDREB1b* (Dehydration response element binding is a cold-inducible transcription factor in *Arabidopsis thaliana*)	Cold resistance	Jin *et al*. 2009
Brachetto	*VvPIP2;4N* gene (PIP-type aquaporin gene)	Water stress resistance	Perrone *et al*. 2012

antifreeze proteins. They analyzed amino acids and found higher levels of alanine (approximately 14 % higher than in the control plants) in a transformed grapevine, but cold tolerance was not evaluated in these studies.

Breeding for abiotic and biotic stress tolerance is breeding for yield. However, the modification of genes involved in other processes - such as root development, flower production, and fruit set - may also be a strategy to increase yield. In grapevine, an increase in the number of flowers and berries was reported by Mezzetti *et al*. (2002a) and Constantini *et al*. (2007) in 'Thompson Seedless' and 'Silcora' transformed with *DefH9-iaaM.* Whereas in the first study the number of flowers was almost doubled in transgenic plants, with respect to the controls, in the second flower number increased only slightly. An increase in productivity (number of flowers or fruit size) and parthenocarpy has been obtained in transgenic eggplant (Donzella *et al*. 2000) or strawberry (Mezetti *et al*. 2002b) plants expressing this gene whereas similar productivity was obtained in transgenic tomato and the respective controls by Ficcadenti *et al*. (1999).

With respect to breeding for quality, this can be achieved indirectly; for instance, when obtaining resistance to a fungus. However, breeding specifically for quality is difficult because this is a complex trait that includes external and internal parameters that are also influenced by the climatic conditions and cultural practices. Therefore, more knowledge is needed to modify grapevine quality with precision by genetic transformation. For instance, MADS-box genes encode transcription factors that are associated with numerous developmental processes - including induction of flowering, specification of inflorescence and flower meristems, establishment of flower organ identity, and regulation of fruit, seed, and embryo development. Recently, Grimplet *et al*. (2016) identified a total of 90 MADS-box genes in the grapevine reference genome. An important berry quality trait is the sugar composition (glucose and fructose in the vacuole of flesh cells). Sugar signaling in grape is concerned mainly with the regulation of anthocyanin biosynthesis and sugar transport, but also with other major processes such as cell growth (Davies *et al*. 2012, Lecourieux 2014). Volatile and non-volatile terpenoids - that greatly influence the varietal character of grapes and subsequently of wine (Lund and Bohlmann 2006) – are other candidates for quality modification. Polyphenolics (flavonoids and non-flavonoids) also contribute to the taste, astringency, color, and mouthfeel of wine (Lund and Bohlmann 2006). Successfully, Thomas and Scott (2001) transformed a seedless cultivar with a UDP:flavonoid 3-*O*-glucosyltransferase (UFGT), to control the color development of grape berries.

Conclusion

Up to now, the breeding of grapevine through genetic transformation has been mainly focused on biotic stress resistance, mainly to fungi and viruses. Other attempts have been related to cold tolerance and modification of berry color. The majority of these studies met with little success due to the difficulty in regenerating plants, the availability of few characterized genes, and/or the quantitative character of the trait. Also, in the majority of these studies, a test of the theoretical resistance obtained in transformed plants was not reported. Comparing the efficiency of genetic transformation among the published studies is really difficult and imprecise because different cultivars, as well as distinct protocols for transformation and regeneration in selective conditions, were used. However, based on the reported work, we can conclude that *Agrobacterium*-mediated transformation is the method of choice for stable transformation of grapevine and that most of the research performed to date has concentrated on 'Chardonnay' and 'Thompson Seedless'. Among the selective agents, *nptII* was the preferred gene for selection despite the sensitivity to kanamycin of some cultivars, for which the concentrations and time of application need to be adjusted to achieve regeneration. When using kanamycin and hygromycin as selective agents, a stepwise procedure seems the most appropriate to obtain regeneration of transgenics, although the regeneration of escapes together with plants that have integrated the transgene is common.

Currently, a great increment in grapevine breeding efforts is expected due to the greatly increased knowledge of gene function and regulation, and of the new promoters and technologies for gene editing, transfer, and selection (Dutt *et al.* 2014, Bortesi and Fischer 2015). Therefore, new or modified protocols that facilitate the recovery of a large number of plants in a broad number of grapevine cultivars and rootstocks, in order to select those with a single integration and correct expression, are required.

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