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DNA Transfer and Gene Expression in Transgenic Grapes

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Introduction

The Old World species, *Vitis vinifera*, is the grape of antiquity often mentioned in the Bible. Most table, wine, and raisin grapes are produced from this variety, which originated in the regions between the south of the Caspian and Black seas in Asia Minor (Winkler *et al.*, 1974). It was probably in Northern Iran or Armenia where the grapevine was found growing wild 8000 years ago. More comprehensive data were lost over generations but masses of crashed grape pips, stems, and skins have been found by paleontologists indicating that wine and table-grapes were widely known and highly popular in distant times. During the centuries grape cultivars were carried from region to region by civilizations. Wine was made in Egypt in the middle of the 4th millennium BC; the hieroglyphics of ancient Babylon contain references to wine and table grapes: fermented beverages were made in China before the year 2000 BC; and the Bible contains many references to the cultivation of the vine in Israel (Zohary and Spiegel-Roy, 1975; Zohary and Hopf, 1988).

Today, vineyards occupy more than 10 million hectares throughout the world, making grapes the world's most widely grown fruit crop. World production of grapes is in excess of 65 million metric tonnes, exceeding the production of all other temperate fruits, and surpassed only by *Citrus* and banana among all other fruit crops around the world (FAO Production Yearbook, 1990). While the major portion is consumed as wine and spirits (60%), grapes are also used in quantity as fresh fruit and dried as raisins. Vine growing is based primarily on traditional cultivars perpetuated for centuries by vegetative propagation. Cabernet Sauvignon (from Bordeaux) and Chardonnay (from Burgundy) are examples of vine grape of pre-Roman and Roman

Abbreviations: ABA, abscisic acid; ArMV, arabis mosaic virus; BAP, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; CP, coat protein; GCMV, grapevine chrome mosaic virus; GFLV, grape fanleaf virus; GUS, β-glucuronidase; LTP, lipid transfer protein; MS, Murashige and Skoog (basal medium); NOA, β-naphthoxyacetic acid; NPTII, neomycin phosphotransferase II; *nptII*, gene coding for neomycin phosphotransferase II; TomRSV, tomato ringspot virus; *uidA* , gene coding for β-glucuronidase.

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origin that are still grown extensively. General references for the grape vine, its taxonomy and history can be found in sundry reviews (e.g. Winkler *et al.*, 1974; Allewelt and Posimgham, 1988).

During the second half of the 19th Century, devastating grape diseases were introduced from America to Europe by exported American grape-plants: powdery mildew in 1852, phylloxera in 1863, downy mildew in 1878 and black rot in 1885. Some of these diseases still remain a major threat to the grape industry world-wide, requiring continuous treatment by fungicides or time and money consuming grafting procedures. It is rather puzzling, that this ancient species is among the most recalcitrant species for genetic transformation and was only recently successfully transformed. Nevertheless, in the near future, with the aid of recent advances in genetic engineering, it is very likely that disease resistance, as well as many other characters, will be introduced into grapevine via transformation technologies.

The importance of genetic engineering to grape breeding

The production of new grape cultivars by conventional breeding is a complex and time consuming process. The fact that breeding methods require a cross to be made between parent plants necessitates the interaction and sorting of two genomes. Progeny of grapevine combine both the positive and negative traits from each parent. Undesirable characters are eventually identified during growth and testing and undesirable seedlings are discarded. In grape, the final assessment of progeny performance is made in relation to the quality and quantity of the fruit produced. Moreover, grapes are highly heterozygous and the characters which constitute a good cultivar are polygenic in their inheritance. Thus, the probability of recombining in a hybrid the set of genes that determine the essential properties of a given cultivar is very low.

Unfortunately, under common horticultural practice, most grape seedlings undergo a juvenile period of about two to three years, before fruit is produced consistently. Reliable assessment of the results of a particular grape breeding strategy may not be possible up to many years after the cross has been made. Given this significant investment of time, the most economical breeding strategy would be to transfer individual traits as single genes into an already available and desirable genetic background. In this case, only the desired trait would be transferred, with minimum disturbance to the original genome.

Embryogenesis in grapes

A prerequisite for achieving an efficient and synchronous transformation system for grape is the establishment of a highly regenerative embryogenic cell suspension suitable for *Agrobacterium* or biolistic mediated transformation. The type and quality of this cell suspension is probably the key factor in enabling successful transformation. The terms quality and type refer not only to its morphogenetic potential but also to its suitability for the transformation vehicle (e.g. *Agrobacterium* or biolistic) as will be discussed later. Due to the importance of embryogenic cell lines for grape transformation, this chapter will review some of the recent developments in this field.

Somatic embryogenesis was first reported for *V. vinifera* (Mullins and Srinivasan,

1976) and for *V. thunbergii* by (Hirabayashi *et al.*, 1976). Unfertilized ovules of Cabernet Sauvignon were cultured in a liquid medium with NOA and BAP (Mullins and Srinivasan, 1976). Proliferating callus eventually gave rise to somatic embryos. The technique was later refined and extended to further genotypes (Srinivasan and Mullins, 1980). Krul and Worley (1977) documented somatic embryogenesis from calli of leaf, petiole and stem segments of the inter-specific hybrid 'Seyval'. The production of somatic embryos from anther-derived tissues has been achieved in numerous cases (Rajasekaran and Mullins, 1979; Rajasekaran and Mullins, 1983a, 1983b; Bouquet *et al.*, 1982). The ability to regenerate from sporophytic anther tissues is highly dependent on both genotype and flower type; hormonal alteration of flower type resulted in a loss of regenerative capacity (Rajasekaran and Mullins, 1983b). Casein hydrolysate, glutamine and adenine have been used to increase embryoid production from cultured anthers of 'Cabernet Sauvignon' (Mauro *et al.*, 1986). Anthers were most responsive when collected near the first pollen mitosis. Chilling of the unopened flowers for 48–72 hours was found to promote the development of embryogenic type of callus (Perl *et al.*, 1995). Both ovary and anther tissues were suitable explants for the induction of long-term embryogenic cultures of *V. acerifolia* (Gray and Mortensen, 1987). This culture has remained embryogenic for 6 years (Gray and Meredith, 1992). The detailed review of Gray and Meredith (1992) describes the different protocols utilized. This review indicates that the phenomenon of embryogenesis is well established in several grape species, but response among cultivated genotypes varies greatly. The rate of initiation of embryogenic cultures is often low. Yet, recent studies, cited below, have been responsible for the extension of embryogenesis to a growing list of cultivars.

Somatic embryogenesis has now been demonstrated with White Riesling using BAP and thidiazuron treatments along with explant chilling (Harst-Langenbucher and Alleweldt, 1993). Bouquet (1989) reported that somatic embryos and plantlets have been obtained from 22 genotypes of *V. vinifera* (14 wine and 8 table grapes), 4 interspecific hybrids, and 12 rootstocks.

Although anther and ovule tissues have most often been used for the induction of embryogenic cultures, other tissues have been used successfully as well. Callus derived from leaf tissue of a Japanese cultivar has produced somatic embryos for over 2 years (Matsuta and Hirabayashi, 1989). Leaf tissue was also used successfully to induce embryogenic cultures from two *V. rupestris* genotypes as well as 'Cabernet Sauvignon' (Stamp and Meredith, 1988a). Zygotic embryos were first reported to be regenerable by Stamp and Meredith (1988b). Embryogenic cultures were induced from *V. acerifolia*, as well as from *V. vinifera* cultivars Chardonnay, French Colombard, Grenache, and White Riesling.

Germination of somatic embryos may be problematic, as has been reviewed in detail by Gray and Meredith (1992) and Torregrosa (1995). A detailed study of this problem determined that many somatic embryos have abnormal or missing shoot apices (Faure, 1990). The suggestion was made that the high humidity conditions found *in vitro* may be unfavourable to germination and may result in embryos with impermeable suberized surface layers. The blockage of conversion of 'abnormal' plants into morphological 'normal' germinating plantlets was previously studied. For some genotypes, chilling (Rajasekaran and Mullins, 1983a), section of cotyledons (Mauro *et al.*, 1986), addition of cytokinins (Gray, 1989), or dehydration (Gray,

1989), improved to some extent the conversion rates. Goebel-Tourand *et al.* (1993) studied the effects of ABA, BAP and zeatin in the culture medium of somatic embryogenesis in cultivars Chardonnay and 41B. Coutos-Thevenot *et al.* (1992b, 1992c) utilized an embryogenic cell suspension of the rootstock 41B as a model system to study the effects of extracellular proteins on embryo development and germination. These suspensions were routinely cultured in the presence of auxin. In order to induce embryo formation and subsequent germination, the cells were transferred to auxin-free medium. Somatic embryos were induced but these embryos were usually arrested at the heart stage of development. Daily subcultures of the embryos to fresh medium were able to promote embryo development indicating the presence of inhibitors excreted into the culture medium (Coutos-Thevenot *et al.*, 1992b). Moreover, when the extracellular protein patterns of embryogenic and non-embryogenic situations were compared, specific proteins and glycoproteins were identified and were found to have a major influence on embryo differentiation (Coutos-Thevenot *et al.*, 1992c). Protein fractions partially purified by ion exchange chromatography caused both an early inhibition of embryogenesis as well as stimulation of secondary embryogenesis. The addition of trypsin increased the rate of embryo development, while the protease inhibitor aprotinin inhibited development and arrest embryos at the globular and heart stages (Maes *et al.*, 1997). Together, these results provide evidence that extracellular proteins modulate somatic embryogenesis and suggest that an extracellular proteolytic mechanism could be implicated in grape somatic embryogenesis (Maes *et al.*, 1997).

Perl *et al.* (1995) have developed a regeneration protocol that enabled a transfer from a differentiated stage to a dedifferentiated stage, and vice versa. Somatic embryogenesis and subsequent diploid plants have been obtained from anthers of *V. vinifera* 4 commercial seedless cultivars. Anthers produce embryogenic calli when cultured on MS medium supplemented with 2,4-D and BAP. Embryos were formed and maintained upon transfer to a MS medium supplemented with IASP and NOA. A synergistic effect was observed while combining these two auxins. Re-callusing was achieved when a single embryo was transferred to MS medium supplemented with IASP and 2,4-D. ABA was found to play a major role in the long term maintenance of this callus. Germination and plantlet formation was characterized by a high frequency of abnormal vitrified plants. Conversion into morphological normal plants was achieved by rooting the abnormal plantlets on MS medium supplemented with α-naphthalenacetic acid. A list of the different embryogenic cell lines that are currently utilized for transformation experiments is provided in *Table 1*.

Methods for transforming grape

MICROPROJECTILE BOMBARDMENT

Since Sanford and co-workers first developed the biolistic transformation system (Klein *et al.*, 1987), research has flourished in developing this system for use with many species. In the last few years, biolistic gene delivery has been used to transform nuclear genomes in a very diverse range of organisms and organelle genomes (for reviews, see Sanford, 1990a, 1990b). This includes bacteria, yeast and other fungi, algae such as *Chlamydomonas*, animal cells, intact animal organs and higher plants

Table 1. Embryogenic cell suspensions currently utilized for transformation experiments

Cultivar designation	Genetic origin	Explant origin	Stock location	Reference
Chasselas	(*V. vinifera*)	Anthers	Swiss	Spielmann *et al.*, (unpublished).
Fercal	(Hybrid × 333EM)	Anthers	France	Mauro *et al.*, (unpublished)
Richter 99	(*V. rupestris* × *V. Berlandieri*)	Anthers	France	Cobanov *et al.*, (unpublished)
Richter 110	(*V. rupestris* × *V. Berlandieri*)	Anthers	France	Mauro *et al.*, 1994
Richter 110	(*V. rupestris* × *V. Berlandieri*)	Anthers	Swiss	Spielmann *et al.*, (unpublished)
Richter 110	(*V. rupestris* × *V. Berlandieri*)	Anthers	France	Le Gall *et al.*, 1994
SO4	(*V. berlandieri* × *V. riparia*)	Anthers	France	Mauro *et al.*, 1995b
St-George du Lot	(*V. rupestris*)	Anthers	Swiss	Spielmann *et al.*, (unpublished)
St-George du Lot	(*V. rupestris*)	Anthers	France	Krastanova *et al.*, 1995
V. rupestris		Petiole	Italy	Martinelli *et al.*, 1994
41B	(*V. vinifera* × *V. berlandieri*)	Anthers	France	Mauro *et al.*, 1995b
1103P	(*V. rupestris* × *V. Berlandieri*)	Anthers	France	Cobanov *et al.*, (unpublished)
3309 Couderc	(*V. rupestris* × *V. riparia*)	Anthers	Swiss	Spielmann *et al.*, unpublished)
Chancellor	(*Vitis* L. hybrid)	Anthers	USA	Kikkert *et al.*, 1996b
Chardonnay	(*V. vinifera*)	Anthers	France	Mauro *et al.*, 1994
Chardonnay	(*V. vinifera*)	Anthers	USA	Kikkert *et al.*, 1996a
Concord	(*V. vinifera*)	Anthers	USA	Kikkert *et al.*, 1996a
Gamay	(*V. vinifera*)	Anthers	Swiss	Spielmann *et al.*, (unpublished)
Grenache noir	(*V. vinifera*)	Anthers	France	Faure *et al.*, 1996
Koshusanjaku	(*V. vinifera*)	Leaves	Japan	Nakano *et al.*, 1994
Merlot	(*V. vinifera*)	Anthers	USA	Kikkert *et al.*, 1996a
Niagra	(*V. vinifera*)	Anthers	USA	Kikkert *et al.*, 1996a
Pinot noir	(*V. vinifera*)	Anthers	USA	Kikkert *et al.*, 1996a
Ugni blanc	(*V. vinifera*)	Anthers	France	Coutos-Thevenos *et al.*, (unpublished)
Centennial Seedless	(*V. vinifera*)	Anthers	Israel	Perl *et al.*, 1995
Italia	(*V. vinifera*)	Anthers	Israel	Perl , (unpublished)
Muscat Seedless	(*V. vinifera*)	Leaves	South Africa	Trautmann *et al.*, (unpublished)
Novomuscat	(*V. vinifera*)	Anthers	Israel	Perl *et al.*, 1995
Red Globe	(*V. vinifera*)	Anthers	Israel	Perl , (unpublished)
Rubby Seedless	(*V. vinifera*)	Anthers	Israel	Perl *et al.*, 1995
Superior Seedless	(*V. vinifera*)	Anthers	Israel	Perl *et al.*, 1995
Thompson Seedless	(*V. vinifera*)	Leaves	USA	Scorza *et al.*, 1996
Thompson Seedless	(*V. vinifera*)	Leaves	South Africa	Trautmann *et al.*, (unpublished)
2-19-6	(*V. vinifera*)	Zygotic embryo	USA	Scorza *et al.*, 1995
72-659-2	(*V. vinifera*)	Zygotic embryo	USA	Scorza *et al.*, 1995
69-636-5M	(*V. vinifera*)	Zygotic embryo	USA	Scorza *et al.*, 1995

(Sanford *et al.*, 1993). The successful application of the biolistic transformation process in many species, and the availability of new and improved systems for grapevine regeneration from cultured tissues, made it likely to believe that particle gun transformation of grapevines can be accomplished successfully. Operationally, the biolistic process is subdivided into two stages: (i) coating metal particles (microprojectiles) with nucleic acid, and (ii) accelerating the coated microprojectiles to velocities appropriate for penetration of target cells or tissues without excessive disruption of biological integrity (Sanford, 1990a). As for grapes, the coating process has been modified for factors such as particle size, helium pressure, shooting distance, DNA concentrations, type of solvents and their concentrations (Hébert *et al.*, 1993). In this report, embryogenic suspensions of 'Chancellor' (*Vitis* L. complex interspecific hybrid) were bombarded with tungsten particle coated with plasmid pBI426 encoding GUS and NPTII. Hébert *et al.* (1993) obtained up to 850 transformed callus colonies per plate, 23 days after bombardment. These results demonstrated the biolistic process as a potential tool for achieving stable transformation of grapevines (Hébert *et al.*, 1993). Following the calibrating of the biolistic process for embryogenic grape calli, and improving somatic embryogenesis protocols (Hébert-Soulé *et al.*, 1995), Reisch and colleagues successfully established stable GUS-positive kanamycin resistance transformed 'Chancellor' plants (Kikkert *et al.*, 1996a). With the successful 'Chancellor' model system in place, the current work of Reisch and colleagues is focused today on the use of a chitinase-producing genes to confer fungal disease resistance upon important grapevine cultivars. Endochitinase gene product has been recently shown *in vitro* to inhibit the growth of pathogens that cause *Botrytis* bunch rot and powdery mildew of grapes. Recent results indicate that the chitinase gene was successfully expressed in 'Chancellor', 'Chardonnay', and 'Merlot' regenerating embryos (Kikkert *et al.*, 1996a, Kikkert and Reisch, 1996). At least three 'Chardonnay' and one 'Merlot' plants, that overexpress the endochitinase gene, are currently at the greenhouse for fungal tolerance evaluation (B. Reisch, personal communication).

AGROBACTERIUM RHIZOGENES

Experiments designed to develop a transformation system for grape, utilizing *Agrobacterium rhizogenes*, were recently reported. Genetically transformed grapevine roots of cultivar Grenache were obtained after inoculation of *in vitro* grown whole plants with *A. rhizogenes* (Guellec *et al.*, 1990). In this study, inoculation of young stem or leaf explants resulted in rapid necrogenesis of the plant material and a non-necrotic response was obtained only with older cuttings. Although the plasmid introduced into the grape explants in this study contained *npt*II, conferring kanamycin resistance, the strategy for selection of transformants was based on root development encoded by hairy root T-DNA as a morphogenetic marker. This may be due to the fact, that when transformed and normal roots were exposed to a range of concentrations of kanamycin (0–200 mg/l), no differences in resistance to the antibiotic were seen for any of the cultures, even those displaying NPTII activity (Guellec *et al.*, 1990).

Garibaudo *et al.* (1995) studied the response of different grapevine genotypes ('Nebbiolo', 'Moscato' and 'Barbera') to inoculation with different *A. rhizogenes* strains (A4, 15834, NCPPB 2659 and 8196), utilizing different inoculation tech-

niques. Root production was obtained in all the inoculated sites and for all genotypes tested.

The utilization of *A. rhizogenes* mediated transformed root-cultures for virus production was showed by Lupo *et al.* (1994). *A. rhizogenes* mediated transformation was applied to *V. rupestris, V. riparia, V. vinifera* and several *Vitis* hybrids infected by different phloem-limited viruses such as grapevine fleck virus, grapevine virus A and grapevine virus B (Lupo *et al.*, 1994). All viruses multiplied and persisted in these proliferating root cultures, which were successfully utilized for viral particle purification. *A. rhizogenes* transformed grape roots were proven as a reliable source for virus purification, although the rate of growth was not as satisfactory as compared to transformed *Nicotiana* spp. infected with the same viruses (Lupo *et al.*, 1994). Modification and improvements to overcome these restrictions were recently suggested by Torregrosa and Bouquet (1997). Hairy root cultures of grapevine were obtained when explants were co-inoculated with a mixture of a virulent *A. rhizogenes* strains and a disarmed *A. tumefaciens* strains, harboring the binary vectors pKHG4 and pKVHG 2^{+}. These two plasmids, that contain the genes *npt*II, *hpt* (the gene coding for hygromycin phosphotransferase) and *uid*A, differ by the presence of the gene encoding for the CP of the GCMV. For the cultivar 'Gravesac', 72% of the excised root tips initiated hairy root cultures on hormone free media. Up to 16% of co-transformation was obtained as verified using GUS staining procedures. Although responses varied considerably from clone to clone, kanamycin and hygromycin resistance, as well as CP-GCMV production, were reported for co-transformed roots. Plant regeneration was not achieved, but the authors discuss the possibility to graft *in vitro* transgenic roots to non-transformed shoot systems, in order to permit a novel and rapid testing of the resistance induced by nepovirus CP in root of cultivars which are recalcitrant to *A. tumefaciens*-mediated transformation (Torregrosa and Bouquet, 1997).

Stable transgenic grapevine plants were obtained via somatic embryogenesis after co-cultivation of embryogenic calli with an engineered *A. rhizogenes* strain including both the *npt*II and *uidA* genes, followed by selection of secondary embryos for kanamycin resistance. This study was the first successful report for the production of stable transformed *V. vinifera* L. cultivar Koshusanjaku via *A. rhizogenes* mediated transformation. The *A. rhizogenes* engineered strain, A13/pBI121, harboured both a wild-type Ri plasmid and the binary vector pBI 121 containing the *npt*II and *uidA* genes. Following secondary embryogenesis in the presence of kanamycin, 12 subclones gave rise to transformed plantlets. Southern blots analysis revealed the stable integration of the GUS and kanamycin resistance genes into the plants genome. Differences in phenotype of these transformed plantlets were observed. Some plantlets exhibited a typical Ri-transformed phenotype such as wrinkled leaves and abundant root system. On the other hand, plants from eight subclones looked normal, even though six of them contained a wild-type Ri T-DNA (Nakano and Mil, 1994).

AGROBACTERIUM TUMEFACIENS: A GRAPEVINE DISEASE-AGENT OR A TRANSFORMATION VEHICLE?

The extraordinarily broad host range of *Agrobacterium* and its unique infection characteristics have made this bacterium a very useful tool to introduce foreign genes

into higher plants (Ream, 1989). While most *A. tumefaciens* (biovar 1 isolates) exhibit a wide host range, certain strains isolated from grapevine are oncogenic only on grape cultivars (Thomashow *et al.*, 1980). *A. tumefaciens* biovar 3 (i.e. *A. vitis*) is the causal agent of crown gall and root decay, two important diseases of grapevine in all major viticultural areas (Rodrigues-Palenzuela *et al.*, 1991). Spencer *et al.* (1990) have isolated and identified a phenolic compound which acts as a chemical signal specifically for *A. vitis*. This phenolic compound identified as syringic methyl ester (3,5-dimethoxy-4-hydroxybenzoic acid methyl ester), was found to efficiently induce the *vir*-genes of *A. tumefaciens* co-cultivated with a number of *Vitis* cultivars.

Chemicals, physical and developmental conditions were found to modify the efficiency of tumour formation by *Agrobacterium* in *Vitis* species using *in vitro* cultured plants. Short-day photoperiods preconditioning to assay plants were found to reduce tumor formation. Pretreatment of plants with auxins or cytokinins altered the specificity in various combinations of strains and host genotype (Lowe and Krul, 1991). Nevertheless, Baribault *et al.* (1989) were first to established that biovar 1 strains, commonly used in transformation of herbaceous annual plants, can transform *in vitro* grapevine tissues thus avoiding the need to engineer biovar 3 strains specifically for grapes.

It should be noted that, while for most plant species *Agrobacterium* can be used as a transformation vehicle, for grape it is a bacterial disease as reflected by several grape cultivars that are 'fighting back' and respond by necrogenesis due to a hypersensitive-like reaction. Two different types of necrogenesis have been described for grape following co-cultivation:

(i) A progressive necrogenesis response (hypersensitive-like reaction) observed on grape leaves, petioles and stem pieces inoculated with strains of *A. tumefaciens* originating from biovars 1,2 and 3 (Pu and Goodman, 1992, 1993). This necrogenesis, which was visible 7–14 days after inoculation, was observed using most *Agrobacterium* spp. and was also cultivar specific (Pu and Goodman, 1992). The presence of the T-DNA within the bacterial strain was crucial for necrogenesis induction, since strains that their T-DNA was deleted were neither tumourigenic nor necrogenic (Deng *et al.*, 1995). It had been suggested that the avirulence of the wide host range strain to *Vitis* is due to the hypersensitive response. In this hypersensitive response, *Agrobacterium* infected plant cells are killed at the site of inoculation, but transformation preceded necrogenesis and subsequent cell death (Yanofsky *et al.*, 1985; Pu and Goodman, 1992).

(ii) In recent studies using *in vitro* embryogenic cell lines it has been demonstrated that a totally different type of necrogenesis preceded transformation while utilizing binary vectors in disarmed biovar 1 *Agrobacterium* strains. This cultivar's specific necrogenesis was visible within 2–3 days after co-cultivation, and was not influenced by the presence of the T-DNA since autoclaved *Agrobacterium* was found to induce the same necrotic response. It was surprising to see that biovar 3 strains did *not* induce necrogenesis as did living or autoclaved biovar 1 bacteria (Perl *et al.*, 1996a; Perl *et al.*, in preparation).

AGROBACTERIUM TUMEFACIENS AND APICAL MERISTEM TRANSFORMATION

Grapevine cells in culture were shown to be susceptible to the commonly used biovar 1 strains containing either a binary or co-integrated vector (Baribault *et al.*, 1989). Co-cultivation of cultures with *Agrobacterium* strains bearing either the co-integrated pGV3850:1103neo, or the binary vector pGA474-68, resulted in kanamycin resistance tissue. The stable integration and expression of the neomycin phosphotransferase gene was confirmed by Southern blotting and enzymatic assays. This report established for the first time that biovar 1 strains, commonly used in transformation of herbaceous annual plants, can transform grapevine tissue by the integration of a chimeric *npt*II gene into the grapevine genome.

The first successful report (that was never repeated), on regeneration of transformed grape plants was published in China. Hung *et al.* (1989) obtained seven transgenic plants by infecting apical meristem explants from over 300 cuttings of Cabernet Sauvignon, Chardonnay, Grenache and Riesling with *A. tumefaciens* strain carrying a modified Ti plasmid, pGV3850:1103 neo, which conferred nopaline production and resistance to 100 mg/l kanamycin. Shoot regeneration and subsequent genetic transformation were achieved by co-cultivation on an MS medium supplemented with 10 mg/l BAP.

A similar approach was taken further by Baribault *et al.* (1990) while studying genetic transformation in fragmented shoot apex cultures. Co-culturing of grapevine fragmented shoot apices with various disarmed *Agrobacterium* vectors carrying the *npt*II gene leads to the regeneration of shoots which tolerate low levels of kanamycin (10–25 mg/l). Although such levels are toxic to normal shoots, none of the tolerant shoots has rooted in the presence of kanamycin. Since the construct also contained the visual GUS reporter gene, Baribault and colleagues suggested that the kanamycin resistant shoots were of a chimeric origin and contained both transformed and non-transformed cells. In their study, and contradicting the report of Hung *et al.* (1989), grapevine were found to be very sensitive to kanamycin. A concentration of 5 mg/l was sufficient to stop shoot elongation, rooting and growth of fragmented shoot apices. Thus, rendering it impossible to select kanamycin-resistant grapevine by application of selection pressure during the early stages of culturing fragmented apices following *Agrobacterium* infections. Nevertheless, disregarding selection pressure, apices or fragmented shoots were not considered as the optimal explant for developing an efficient transformation system mainly due to the chimeric origin of transformed shoots obtained by this method.

These first reports on transformation in grapes and the following studies by Mullins *et al.* (1990) and Berres *et al.* (1992), defined grape as a recalcitrant species for gene delivery. Mullins *et al.* (1990), utilizing petiole explants of Cabernet Sauvignon, regenerated only transgenic buds of this cultivar following *A. tumefaciens* transformations. These latter results, which have not been repeated, and the studies of Colby *et al.* (1991) and Colby and Meredith (1990), clearly demonstrated the obstacles in transforming grapes and the necessity of improvement of transformation and selection techniques. Mullins *et al.* (1990) concluded that the main obstacle for grape transformation was not in *Agrobacterium* infectivity or gene integration, but that the main problem was the need to balance the selection requirement for a relatively high concentration of kanamycin, with the inhibitory effects of this antibiotic on shoot

emergence. Anatomical and histological analyses of co-cultivated regenerating leaf explants provided more data supporting the latter assumption. Colby *et al.* (1991) and Colby and Meredith (1990) investigated the compatibility of direct shoot organogenesis with *Agrobacterium*-mediated transformation. No confirmed transgenic shoots were recovered, although 70% to 90% of Thompson Seedless and French Colombard leaf explants produce adventitious shoots. This may be due to the fact that *Agrobacterium* most frequently transforms cells at or below the cut surface of the petiole, a region that never regenerates. These results suggest that the co-cultivation and direct shoot regeneration system, utilizing fragmented shoot apices, leaf blade or petioles, would result at best with some chimerically transformed plantlets. Obviously, it is unsuitable for the routine production of uniformly transformed stable grape plants.

A different approach was taken by Berres *et al.* (1992). A T-6b gene, originated from the grapevine-specific *Agrobacterium* biovar 3 strain Tm4, was found to be involved in growth stimulation probably via involvement in the cytokinin biosynthesis pathway. Transformation experiments were performed with the *Agrobacterium* biovar 1 strain GV3101 containing the T-6b gene in disarmed GUS vector. The T-6b gene was shown to enhance the ratio of transformed versus untransformed cells on stem fragments. Both stem and leaf tissue were efficiently transformed when the wild-type T-6b gene was included in the transferred T-DNA. Although this approach did not result in regeneration of stable transformed plants, it could be used in rapid transformation assays to test the efficiency of improved strains or to test the expression of gene constructs which do not require regeneration.

AGROBACTERIUM TUMEFACIENS AND EMBRYOGENIC CELL-LINE TRANSFORMATION

The difficulties in grape–*Agrobacterium* interactions regarding chimeric transformed plants were mostly solved by the utilization of embryogenic cell lines as the explant for *Agrobacterium* co-cultivation. As mentioned before, the quality of this cell-suspension is the main key factor determining success or failure in obtaining grape transformation. In general, two main types of cell suspension were described. Obviously, both types should be successfully maintained for the long-term without the loss of their morphogenetic potential to regenerate plants. The first type of embryogenic line is composed of fine cells, arrested in a very early pre-embryogenic state. These callus-type cells multiply *in vitro* as the result of the presence of auxins (mainly NOA) in the culture media. As soon as the auxin source is removed, regeneration of embryos and subsequent plantlet germination takes place, usually on a hormone-free medium. When these types of suspensions are co-cultivated with *Agrobacterium*, transformation efficiency is very high and selection, using mainly paromomycin or kanamycin, may start immediately at the end of the co-cultivation stage. Performing the selection procedure in liquid media, with repeated subcultures to fresh antibiotic supplemented media, reduces escapees due to a more efficient selection process and better penetration of the selection agent compared to solid media. The second type of embryogenic culture is composed of embryos arrested in a more advanced stage of development. These cultures multiply by secondary embryogenesis, starting mainly from hypocotyl regions of existing embryos and rarely from cotyledons. Selection following transformation should start only when secondary embryogenesis is visible, which might take

place only weeks following co-cultivation. This type of embryogenic culture is much more difficult to transform due to a lower efficiency of transformation and the tendency of these cultures to response to the presence of *Agrobacterium* in a hypersensitive-like reaction, leading to necrogenesis and subsequent culture death. Using fine-cell suspension embryogenic cultures we have never observed necrogenesis, indicating that this hypersensitive-like reaction is cultivar dependent and developmentally regulated as well (Perl *et al.*, in preparation). A similar system was recently described by Calderon *et al.* (1993, 1994). Cell suspension of *V. vinifera*, treated with a specific fungal elicitor, showed a hypersensitive-like response. This was characterized by cell plasmolysis and was accompanied by localized cell death, which was concomitant with cell culture browning. In addition to these responses, increased amounts of benzoic acid and resveratrol were observed in the treated cells before browning and subsequent cell death. Browning was at least partially due to the formation of resveratrol oxidation products and the activation of polyphenol oxidase and/or peroxidase.

Agrobacterium tumefaciens *mediated Vitis rootstocks transformation*

V. rupestris somatic embryos, capable of secondary embryogenesis, were co-cultivated with *A. tumefaciens* strain LBA 4404 upon transfer to a medium inducing secondary embryogenesis. This specific timing of co-cultivation was found to promote the delivery of the introduced genes into the embryogenic tissue in its active stage of cell division (Martinelli and Mandolino, 1994; Martinelli, 1995). Wounding of embryogenic tissue proved unnecessary for achieving DNA delivery into plant-cell genome. In spite the reports on kanamycin sensitivity of *Vitis* tissues, precocious selection for high levels of kanamycin (100–150 mg/l) were an important factor for successful transformation. Transformed lines retained the activity of the introduced genes for more than three years, during which time they have also maintained their potential for secondary embryogenesis and plant regeneration.

A more comprehensive study on the stability of the expression of the *uid*A gene in these transgenic plants had been conducted in population of transgenic plants regenerated from these somatic embryos. Molecular tests demonstrated no loss of the inserted gene following either the long-term embryo-culture or subsequent plant regeneration (Martinelli and Mandolino, 1996).

A highly important grapevine rootstock, 110 Richter (*V. rupestris* X *V. Berlandieri*), was a target cultivar for several transformation experiments aiming to introduce agronomically important genes (mainly virus resistance). Transgenic plants of 110 Richter were regenerated from embryogenic cultures co-cultivated with a disarmed LBA 4404 strain of *A. tumefaciens*, harboring a binary vector contained chimeric genes for hygromycin resistance (*hpt*), kanamycin resistance (*npt*II), the *uid*A gene and the CP-GCMV (Le-Gall *et al.*, 1994). Nepoviruses are an important group of phytoviruses characterized by isometric particles and transmission by soil-inhabiting nematodes. Some of them have a very important economic impact on grapevine, especially grape fanleaf virus (GFLV) and arabis mosaic virus (ArMV). GCMV has a geographic distribution limited to several central European countries, but in regions where it is widespread, it is very damaging as it induces symptoms, lack of vigour and unfruitfulness as severe as those caused by GFLV (Bouquet, 1993; Le-Gall *et al.*, 1994).

Acetocyringone (50μM) and vaccum infiltration (700 mm Hg, 5 min) were utilized to enhance *Agrobacterium* virulence and attachment to the embryogenic cells respectively. The best rate of transformation was obtained by selection of putative embryogenic tissues grown on a medium containing 16 mg/l hygromycin. High levels of CP-GCMV expression were detected by ELISA of somatic embryos, leaves and roots of transformed plants. These results were confirmed by Western blot, thus indicating that hygromycin resistance can be a reliable selectable marker in grapevine transformation. The other selectable markers (e.g. kanamycin and GUS) were also expressed in the transformed plants, as was confirmed by Southern blot analysis. Theoretically, the obtaining of a resistant rootstock cultivar is sufficient to protect an entire vineyards against infection by a soil-borne virus. However, it is not sure that a CP-mediated protection expressed in a rootstock will protect the grafted vines on the long term from viral infection in the field (Le-Gall *et al.*, 1994; Torregrosa *et al.*, 1994).

The same two cultivars (i.e. 110 Richter and *V. rupestris*) were also the target cultivars for CP-GFLV transformation (Krastanova *et al.*, 1995). An incubation of 30–60 min with *Agrobacterium* enabled the recovery of transformed tissues (embryos at various stages of development, hypocotyls, leaflets) as judged by GUS staining. For incubation times of less than 30 min, the transformation efficiency was very poor and for more than 60 min, the regeneration rate was considerably decreased. All plantlets selected on a medium containing 50 mg/l kanamycin were uniformly blue. Nearly all leaflets harvested from these plants reacted positively in ELISA, but exhibited different levels of expression of the CP gene. The stability of the insertions had been proven by analysis of plantlets obtained after micropropagation of the transgenic plants in the absence of kanamycin selection.

All the above reports clearly demonstrated the possibility of *Agrobacterium* mediated transformation of embryogenic lines. But, the transformation efficiency was found to be low, thus resulting in only a few plants that reached the field evaluation stage (Krastanova *et al.*, 1995).

A highly efficient transformation and regeneration system that gave rise to a high percentage of stably transformed cells, and therefore allows the development of numerous intact transformed plants, was described by Mauro *et al.* (1995b). In this study, the two economically important rootstocks 41B (*V. vinifera* X *V. berlandieri*) and SO4 (*V. berlandieri* X *V. riparia*) , widely grown in Champagne and Cognac areas, were transformed with the CP-GFLV. The target cells for *Agrobacterium* mediated transformation were anther-derived embryogenic calli that were routinely sub-cultured in the presence of maltose and glycerol as the carbon sources and NOA as an auxin source. As soon as the auxin was omitted, regeneration and subsequent embryo germination were efficiently achieved as discussed above. Selection was performed in liquid media containing paromomycin. This antibiotic was added progressively to the culture medium starting at 5 mg/l to a final concentration of 20 mg/l, in order to select antibiotic transformed cells. To select during the regeneration stage in medium devoid of auxin, only 5 mg/l of paromomycin were used. Transformation efficiency was evaluated using the GUS reporter gene during continuous growth of the cells in the presence of paromomycin. The percentage of blue cells fluctuated from 50% up to 97%, having the average ratio of 62.5% for 41B. This percentage increased with further paromomycin selection. Six months after transfor-

mation, the embryogenic suspension contained an average 94% transformed cells! Paromomycin was preferred to kanamycin because of a quicker effect on cell death and thus a more effective selection. For SO4, 2 months after co-cultivation an average of 50% of transformed cells was reached, which seems to be the maximum for this rootstock. This is mainly due to the fact that the embryogenic aggregates are bigger in size for SO4 compared to 41B, leading to a less efficient selection of the transformed cells. GUS assays were also done on heart-shape and torpedo embryos after transfer to regeneration medium. Of the SO4 embryos 25% were completely blue, while this percentage was 90% for 41B!. The presence of the CP gene was tested by both PCR and Southern blots using the CP gene as a probe. One or two insertion bands were detected in most plants. ELISA assays were performed for 200 transgenic plants. Out of 200 plants, 41.5% were positive exhibiting a value of at least two times higher that the control value. Seventeen plants showed level four to five times higher than that of the control (Deloire and Mauro, 1991; Mauro *et al.*, 1994, 1995a, 1995b).

The resistance of the transgenic grapevine to GFLV is currently being investigated in field experiments by employing the natural way of virus infection through nematodes, in a collaborative program of INRA and LVMH (M. Boulay – personal information).

Agrobacterium tumefaciens *mediated* V. vinifera *transformation*

The famous wine *V. vinifera* variety, Chardonnay, was also recently transformed by Mauro *et al.* (1995b). The transformation protocol was similar to the ones previously reported by the same research team for the transformation of 41B and SO4 rootstocks. Embryogenic cell suspensions of Chardonnay were co-cultivated with *Agrobacterium* strains harboring a binary vector containing the CP-GFLV and the NPTII genes. Two months after transformation and subsequent paromomycin selection, an average of 50% of transformed cells was reached. High efficiency of embryo and plant development allows the regeneration of a high number of independent, transformed plants. Selection of transformed embryogenic cells was effective, especially with paromomycin, which induced cell death in non-transformed cells within 2–3 days. However, Chardonnay cells, due to the large embryogenic aggregates, required higher concentration (up to 20 mg/l) for selection.

Table-grape cultivars were also the target of transformation experiments. Transgenic table-grape plants were regenerated from embryos derived from immature zygotic embryos of seedless *V. vinifera* selections. Scorza *et al.* (1995) used a novel approach to promote transformation efficiencies. They combined particle bombardment with *Agrobacterium* mediated transformation. Particle bombardment was used as a wounding method of the embryogenic explants prior to the co-cultivation stage. Scorza and his colleagues (1995) combined their highly proliferated embryogenic cultures with the highly effective particle-wounding/*A. tumefaciens* treatment in order to promote the recovery of transgenic plants. Following co-cultivation for 2 days, cultures were allowed to proliferate for 6 weeks before being placed onto selection medium. Selection was carried out by the addition of 20 mg/l kanamycin for the first 6 weeks and then 40 mg/l for the next 6 weeks of proliferation. Transgenic embryos were identified after 3–5 months under selection and allowed to germinate and develop into rooted plants on Woody Plant Medium (Lloyd and McCown, 1981) containing 1 μM

BAP. Of approximately 300 somatic embryos bombarded (100 of each genotype) 5 distinct transgenic lines were produced. Integration of the *uid*A and *npt*II genes into these grapevines was verified by both PCR and Southern analysis.

Based on these findings, that were obtained by utilizing embryogenic cultures derived from zygotic embryos with unknown commercial identity, Scorza *et al.* (1996) obtained transgenic 'Thompson seedless' plants carrying agriculturally important traits. Somatic embryos, derived from leaves of *in vitro* grown plants, were exposed to *Agrobacterium* harboring either the lytic peptide Shiva-1 or the tomato ringspot virus (TomRSV) CP gene. Somatic embryos were either bombarded with gold microprojectiles and then exposed to *A. tumefaciens* or were exposed to *A. tumefaciens* without prior bombardment. Thirteen plants (obtained with or without pre-bombardment) survived kanamycin selection. PCR analysis, using CP-TomRSV and Shiva-1 primers or Southern blots using the *npt*II gene as a probe, suggested that these 13 plants contained the predicted gene sequences. Due to the low number of plants obtained in this study, it was impossible to determine the importance of the pre-cultivation bombardment treatment. Nevertheless, this study demonstrated the successful transformation of 'Thompson Seedless' a major *V. vinifera* scion cultivar.

While trying to study the biosynthesis, transport, storage and distribution of sugars in the developing and maturing grape berry, Perl *et al.* (1994) have established transgenic grape plants that overexpress invertase, an enzyme that is involved in hexoses accumulation in ripening berries. Embryogenic cell lines of *V. vinifera* cultivar Superior Seedless were established and utilized for transformation experiments. Stable transgenic grape plants resistant to hygromycin were obtained. These plants were transformed with *suc2* , a yeast-derived invertase gene, under the control of a synthetic gibberellin inducible promoter. Plants are currently in greenhouse experiment to determine the yeast invertase activity in transgenic berries following gibberellin spraying.

Very short exposures of Superior Seedless embryogenic calli to diluted cultures of *Agrobacterium* resulted in plant tissue necrosis and subsequent cell death. Antibiotics used for *Agrobacterium* elimination or as plant selectable markers were not responsible for this necrotic response. Thus, it seemed to be oxygen dependent and was correlated with elevated levels of peroxidases activity. Perl *et al.* (1996a) studied the effects of various combinations of antioxidants on the necrotic response during and after grape-*Agrobacterium* co-cultivation. A combination of polyvinylpolypyrrolidone and dithiothreitol was found to improve plant viability. Tissue necrosis was completely inhibited by these antioxidants while *Agrobacterium* virulence was not affected. Antioxidant treatments were the key factor in establishing stable transgenic grape plants resistant to hygromycin or Basta (Perl *et al.*, 1996a, 1996b). Recently it has been found that a 35 kD heat-stable *Agrobacterium* protein is responsible for elicitation of necrogenesis in embryogenic tissues during transformation, indicating a putative harpin protein (Perl *et al.*, in preparation).

Gene expression in transgenic grapes

Field experiments with transgenic grape cultivars, expressing agronomically important traits have been launched during the last two years. Most of these plants are still juvenile and only recently started to grow in the fields. Obviously no data are yet

available on viral or bacterial disease resistance of these plants. The main field experiment involves resistance of transgenic grapevines to GFLV and is performed in a collaborative program of INRA and LVMH (M. Boulay – personal information). *Table 2* summarizes all published studies in which agronomically important traits were introduced into grape via either *Agrobacterium* or microprojectile-mediated transformation.

Patents related to grape embryogenesis and transformation

Grape transformation is mostly an applied research in its nature; thus, some of the results obtained in this field have been documented as patents rather than published research articles. Although very rarely do reviews provide a summary of this kind of information, we would like to describe the current state of the art regarding registered grape embryogenesis and transformation patents.

The followings are patents related to embryogenesis in grapes:

- Differentiation of grape suspension cells in culture was promoted by adding to the culture medium at least one LTP. LTP is used to produce somatic plant embryos from somatic cell cultures *in vitro*. A patent by Boulay *et al.* (1992) claims rights for plants produced by this process, to any protein having LTP activity, and to its corresponding genes. In subsequent patents originated from the same research group (Coutos-Thevenot *et al.*, 1992a, 1993b; Mauro *et al.*, 1992), a method aimed to stabilize a culture of proembryogenic cellular aggregates is described. The patents describe the maintenance of proembryogenic vine cells in conditions inhibiting cell differentiation into embryos while permitting mitosis. On the other hand, the patents describe a process for developing embryos from such strains and subsequent plant regeneration. This new cell strain can regenerate at least 100 embryos per mg of cells, as compared with 1 embryo per mg for known cell strains. The stabilized cultures are highly suitable for transformation.
- Production of secondary embryos from a culture of primary somatic embryos was stimulated by adding embryogenesis-inhibiting protein. This protein was identified and extracted from cultures of developmentally blocked embryogenic cultures. When added to the culture medium, it was found to inhibit primary embryogenesis but to stimulate secondary embryogenesis from primary embryos (Coutos-Thevenot *et al.*, 1993a; Maes *et al.*, 1993).
- Different results, related to embryogenesis, were described and patented by Krul (1982, 1987). These patents include the isolation of single nodes selected from plants of the cultivar Seyval, developing of plantlets, induction of embryogenesis on these *in vitro* leaves following exposure to alternating photoperiods, and adding effective growth regulators. These procedures are mainly orientated for grapevine propagation through rapid clonal multiplication, isolation of mutants and insertion of gene vectors.
- Similar explants are utilized by Marchenko (1989) in the patent describing the production of genetically altered vine plants. This study utilized internodes or leaves as cuttings which were then cultivated in the presence of 2,4-D and BAP to grow calli and subsequently embryos.

Table 2. The sate of the art regarding agronomically important traits currently introduced into grape cultivars via *Agrobacterium* or the biolistic apparatus

Target cultivars	Strain	Selection	Traits of interest	Gene utilized	Reference
***Agrobacterium*-mediated transformation**					
Chardonnay	LBA 4404	Par	GFLV resistance	CP	Mauro *et al.*, 1995b
Chasselas	LBA 4404	Kan, Par	GFLV, ArMV resistance	CP, Oligo. synthase, Replicase	Spielmann *et al.*, PC
Gamay	LBA 4404	Kan, Par	GFLV, ArMV resistance	CP, Oligo. synthase, Replicase	Spielmann *et al.*, PC
Gamay cells	–	Kan	Ethylene production	ACC oxidase	Ayub *et al.*, 1993
Red Globe	EHA 101	Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Perl, unpublished
Red Globe	EHA 101	Kan	Fruit quality	RNase	Perl, unpublished
St. George	LBA4404	Kan, Par	GFLV, ArMV resistance	CP, Oligo. synthase, Replicase	Spielmann *et al.*, PC
SO4	LBA 4404	Par	GFLV resistance	CP	Mauro *et al.*, 1995b
Superior Seedless	LBA 4404	Basta/hyg	Herbicide resistance	*bar*	Perl *et al.*, 1996a
Superior Seedless	EHA 101	Basta/hyg	Herbicide resistance	*bar*	Perl *et al.*, 1996a
Superior Seedless	GVE3101	hyg	Fruit quality	Yeast *sucII* invertase	Perl *et al.*, 1994
Thompson Seedless	EHA 101	Kan	TomRSV resistance	CP	Scorza *et al.*, 1996
Thompson Seedless	EHA 101	Kan	Bacterial disease resistance	Shiva-1 lytic peptide	Scorza *et al.*, 1996
V. rupestris	LBA 4404	Kan	GFLV resistance	CP	Krastanova *et al.*, 1995
41B	LBA 4404	Par	GFLV resistance	CP	Mauro *et al.*, 1995b
110 Richter	LBA 4404	Hyg, Kan	GCMV resistance	CP	Torregrosa *et al.*, 1995, Le Gall *et al.*, 1996
110 Richter	LBA 4404	Kan	GFLV resistance	CP	Krastanova *et al.*, 1995
Gene gun-mediated transformation					
Concord		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert *et al.*, 1996a
Chardonnay		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert *et al.*, 1996a
Chancellor		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert and Reisch, 1996
Merlot		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert *et al.*, 1996a

Abbreviations: ArMV, arabis mosaic virus; CP, coat protein; Hyg, hygromycin; GCMV , chrome mosaic nepovirus; GFLV, grapevine fanleaf virus; Kan, kanamycin; Oligo. synthase – 2,5 Oligoadenylate synthase; Par, paromomycin; PC, personal communication; TomRSV, tomato ringspot virus.

Concluding remarks

During the last years, biological and genetic barriers have been overcome step by step by modern biotechnology and the advancing technology of genetic engineering . The past three years have seen rapid advances in the application of gene transfer technology to grapes. In the near future, science will have in hand the technologies to genetically modify most agricultural important crop species. Thus, it seems that the scientific community and biotechnological companies are currently facing their biggest and final challenge: how to assure the sceptical public of the value of transgenic crops in general and grape in particular. In grape, where ancient cultivars and tradition have gone hand in hand for centuries, this issue is of particular interest, as was recently exhibited by *The Wall Street Journal*: . . .'Geneticist manipulates wines; Will the industry pop its cork?' . . . (December 28, 1994). Biotechnology in grapes will help to minimize the utilization of pesticides and fungicides, reduce the cost of production, and permit continued productivity in vineyards hit with virus diseases. Within 5–10 years, when transgenic vines and rootstocks are likely to become commercially available, the public should have become more accustomed to the consumption and use of transgenic fruits, vegetables and fiber crops. There are already transgenic tomatoes, squash, potatoes and cotton on the market. Elite transgenic improved grape varieties should not be far behind (Kikkert and Reisch, 1996).

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