Transgenic Plant Journal ©2007 Global Science Books

Transgenic Grapevines

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ABSTRACT

Grapevine (*Vitis vinifera*) is one of the most valuable horticultural crops in the world. Traditionally the geographic distribution was in areas with a Mediterranean climate, but in the last decades the production area expanded to temperate areas in the Northern and Southern hemispheres. A long-term objective of grapevine breeding is to increase cultivar resistance to plant pathogens resulting in the reduced use of labour and fungicides with benefits for winegrowers, consumers and the environment. To enhance the potential of existing cultivars as well as to develop new cultivars resistant to biotic and abiotic stress factors, to overcome limiting climatic conditions, to improve traits of economic value like colour, reduced browning, improved yield, by taking advantage of the increasing knowledge available in grapevine genetics, genetic transformation is a key technology. In this review the technical approaches for creating transgenic grapevines through transformation, selection and regeneration will be discussed, beginning with the approach for virus resistance breeding, since it provided the first transgenic plants with agronomically interesting traits. The current stage of transgenic grapes with constructs conferring resistance to fungi and bacteria will be highlighted. Finally, an outlook on thoughts about new construct design in the view of discussions about safety aspects raised in public perception and hindering acceptance will be presented.

Keywords: resistance to biotic and abiotic stress, *Agrobacterium* mediated transformation, molecular characterization, public acceptance
Abbreviations: AMPs, antimicrobial peptides; **ARMG**, antibiotic resistance marker genes; **ArMV**, Arabis Mosaic Virus; **EFSA**, European Food Safety Agency; **GFLV**, Grapevine Fanleaf Virus; **GVA**, Grapevine Virus A; **GVB**, Grapevine Virus B; **GCMV**, Grapevine Chrome Mosaic Virus; ***npt*II** gene, neomycin phospho-transferase II; **PD**, Pierce's disease; **PG**, polygalacturonase; **PGIP**, polygalacturonase-inhibiting protein; **PGTS**, post transcriptional gene silencing; **T-DNA**, transfer DNA

CONTENTS

LIFE CYCLE AND CULTIVATION OF GRAPEVINE

Grapevines were developed around the Mediterranean basin and since the Early Bronze Age have contributed significantly to food production (Zohary and Hopf 1994). Grapevine thrives in Mediterranean-type environments, but can tolerate cooler and more humid conditions. Wild grape, *Vitis vinifera* ssp. *sylvestris* are widely distributed from the Atlantic coast to Tadzhikistan and the Western Himalayas. They are primarily forest climbers and seem to be native to the humid and mild forest area south of the Caspian Sea and the Southern coast of the Black Sea. The rapid domestication of the grapevine was supported by the manyfold uses for production of table fruit, wine, juice and raisins (Alleweldt *et al*. 1990). All known *Vitis* sp. have 2n = 38 chromosomes, except the genus *Muscadinia* (40 chromosomes), can be crossed and their F_1 hybrids are vigorous and fertile.

Viticulture is based on the maintenance of vegetatively propagated clones either by rooting of winter dormant twigs or by grafting (Olmo 1976). An inventory revealed the existence of more than 14,000 cultivars, from which only about half are maintained in collections or grown commercially (Alleweldt and Possingham 1888). Several species of grape and their hybrids are currently cultivated (Einset and Pratt 1975). *Vitis vinifera* is the sole Mediterranean representative of the genus *Vitis*, comprising several dozen species (Mullins *et al*. 1992), and cultivars of this species account for the vast majority of world production.

Several wild species native to America have been used either as additional genetic sources for breeding of new cultivars or as hardy stocks for grafting, or for nematode and phylloxera resistance respectively (Gray and Meredith 1992) (**Table 1**). Complex crosses between *Vitis vinifera*

Received: 15 March, 2007. Accepted: 4 May, 2007.

Invited Review

Table 1 Main germplasm for tolerance or resistance to pests and diseases (based on Galet 1979).

Pest (disease)	Genetic resources
Dactylosphaera vitifolii (phylloxera)	*V. riparia* Mich., *V. rupestris* Scheele, *V. berlandieri* Pl., *V. cinerea* Engelm. *V. champini* Pl., *V. rotundifolia* Mich.
Meloidogyne spp. (root-knot nematodes)	*V. champini* Pl., *V. rotundifolia* Mich. *V berlandieri* Planchon and *V. candicans* Engelm
Xiphinema sp. (dagger nematodes)	*V. rufotomentosa* Small
A. tumefaciens (crown gall)	*V. amurensis* Rupr., *V. labrusca* Mich.
Xylella fastidiosa (Pierce's disease)	*V. rotundifolia* Mich., *V. simpsoni* Muns. *V. shuttleworthii* House, *V. aestivalis* Michaux *V. rupestris* Scheele
Botrytis cinerea (bunch rot)	*V. vinifera* L., *V. riparia* Mich., *V. rupestris* Scheele, *V. rotundifolia* Mich.
Plasmopara viticola (downy mildew)	*V. riparia* Mich., *V. rupestris* Scheele, *V. lincecumii* Buckl., *V. labrusca* Mich. *V. amurensis* Rupr., *V. rotundifolia* Mich., *V. cordifolia*
Uncinula necator (powdery mildew)	*V. aestivalis* Mich., *V. cinerea* Engelm., *V. riparia* Mich., *V. berlandieri* Pl., *V. amurensis* Rupr., *V. rotundifolia* Mich.

and American native species yielded disease resistant French-American hybrids, used for wine production (Olmo 1976).

Given their nutritional and dietetic value, grapevines contribute considerably to an improved world food production and human nutrition. The Annual Production of grapes in 2004 reached 65,486,235 metric tons (FAOSTAT 2004), exceeding the production of any other temperate fruit crop. Besides, in value grape surpasses all other fruit crops, due to its widespread multiple uses. Storage of elaborated products for prolonged periods stimulated the development of international trading and wine production represents the ultimate value-added use of a fruit crop (Gray and Meredith 1992).

BREEDING OBJECTIVES FOR GRAPEVINE

Although many *Vitis vinifera* cultivars are either native species, sports of chance seedlings, today controlled hybridization is used for variety breeding (Einset and Pratt 1975; Alleweldt and Possingham 1988). While desirable traits differ for rootstocks and scion cultivars, breeding objectives for grapevines aim at the development of adapted cultivars with superior vigour, yield and quality.

Seedlessness represents a desirable trait in grape breeding and may occur either through a) parthenocarpy, where fruit set and development occur without pollination, or through b) stenospermocarpy, where fruit set requires fertilization, but ovules cease to grow normally and do not develop into seeds. Parthenocarpic cultivars can only be used as pollen parents in breeding programmes, while stenospermocarpy allows breeding through embryo rescue techniques (Emershad and Ramming 1984).

Current grapevine production faces problems with biotic and abiotic stress factors during production, harvest and storage and increased demands from the side of the consumers and the food industry. Adverse effects may appear through factors affecting primary production, internal quality traits or final modifications.

Grapevine is worldwide seriously affected by viral diseases (Bovey and Martelli 1986). Accurate global figures for crop losses due to viruses are not available, but it is generally accepted that losses due to viruses are second only to fungi (Matthews 1991). Since no direct control measures are available, preventive ones, such as quarantine and the use of virus-free propagating stock, have been used to control some virus diseases, and tolerant or resistant cultivars are used to manage others.

Resistance to abiotic stress factors

Grape production has traditionally been associated with regions between 20° and 51° N latitude, however today grapes are grown in temperate regions in the northern and southern hermispheres, and even new adapted cultivars are being grown in tropical and humid climates (Alleweldt and Possingham 1988). Climatic factors, e.g. low temperatures, drought, and edaphic conditions further limit grapevine production. Abiotic stress factors affecting the cultivation of grapevine include winter frost, drought, wind, chlorosis, salinity, and valuable germplasm for tolerance or resistance to abiotic stress factors exists for conventional breeding programmes (Alleweldt *et al*. 1990).

Resistance to biotic stress factors

Grape is subject to an array of diseases caused by bacteria, fungi phytoplasmas, nematodes and viruses (Bovey and Martelli 1986). Insect pests can transmit some of the causative agents, e.g. leafhoppers carry *Xylella fastidiosa*, while nematodes of the genera *Xiphinema* and *Longidorus* transmit nepoviruses, or cause damage directly, e.g. *Phylloxera* and the grape root borer. During the last 50 years, management of fungal pathogens has relied heavily upon the use of synthetic chemicals (Rosslenbroich and Stuebler 2000), not regarded as sustainable, because of the relative ease with which fungicide-resistant strains emerging within vineyard populations (Leroux 2004) and increasing public discussion about pesticides and human and environmental health (Spadoro and Gullino 2005; Elmer and Reglinski 2006). A long-term objective of grapevine breeding is to increase cultivar resistance to plant pathogens resulting in the reduced use of labour and fungicides with benefits for winegrowers, consumers and the environment.

LIMITATIONS OF CONVENTIONAL BREEDING PROGRAMMES

Grapevine has a long life cycle with a juvenile period ranging from 1 to 6 years (Einset and Pratt 1975). Therefore, the breeding cycle for the development of new grapevine cultivars is difficult and time consuming, i.e. around 10 years. Compared to other crops, introgressing specific resistance genes into commercial cultivars by hybridization is not easily accomplished, since the long life cycle of grape, combined with heterozygozity and inbreeding depression make backcrossing and recurrent selection very difficult (Alleweldt ad Possingham 1988). In most cases only the F_1 generation can be selected.

In conventional breeding programmes existing sources of resistance are used, the overall objective being to combine disease resistance of one parent with quality traits of the other. Since *Vitis vinifera* is considered for agronomically interesting characteristics, but is known to be susceptible to many pathogens, the hybridizations with resistant species (**Table 1**) has been the only method available to produce resistant cultivars (Einset and Pratt 1975).

Some Middle-Eastern *V. vinifera* and *Vitis* species belonging to the sub-genus *Muscadinia* show a good level of resistance to *Xiphinema index* and/or to GFLV, but this does not completely exclude viral infection (Walker and Meredith 1990; Staudt and Weischer 1992). Until now, traditional breeding methods have not yet allowed the release of new cultivars truly resistant to GFLV and/or its vector. The disease was controlled by soil disinfection using nematicides, currently forbidden in many countries, due to high toxicity of the chemicals, which requires alternative control approaches.

TRANSFORMATION, SELECTION AND REGENERATION APPROACHES

Established grapevine cultivars are an integral part of the economy and cannot be replaced easily by newly bred cultivars. Through the application of directed genetic modification making new traits available, breeding steps requiring less time, desired traits can be directly introduced into established cultivars. Possible applications of genetic transformation included in the early days the integration of virus resistance genes, mainly viral coat protein sequences, resistance to insects by the integration of *Bacillus thuringiensis*-genes and to herbicides (Gray and Meredith 1992).

Genetic transformation is a key technology to enhance the potential of existing cultivars as well as to develop new cultivars resistant to biotic and abiotic stress factors, to overcome limiting climatic conditions, to improve traits of economic value like colour, reduced browning, improved yield, by taking advantage of the increasing knowledge available in grapevine genetics.

Grapevines were considered recalcitrant material for molecular biology techniques, including genetic transformation (Gray and Meredith 1992). The production of transgenic grapevines was first reported by Mullins *et al*. (1990) with the first GUS-positive grapevine rootstock *V. rupestris* 'St. George'.

The methods mainly applied for grapevine transformation include: a) using biological vectors, e.g. *Agrobacterium*-mediated transformation and b) non-biological vector systems, especially micro-bombardment. The use of *Agrobacterium* as a biological vector benefits from the fact that grapevines are within its host range, although biovar 1 strains are generally employed for transformation (Gray and Meredith 1992). Furthermore this transformation method targets the T-DNA to the nucleus, leading to a stable integration into the host DNA, frequently in low copy numbers when compared to that obtained with biolistics (Mehlenbacher 1995). However, particle bombardment has also been efficiently applied in the transformation of grapevine (Scorza *et al*. 1996). The regeneration of transformed plantlets is recognized for many years as a major bottleneck in the transformation of grapevine cultivars. Although Haberlandt postulated the totipotency of all plant cells, culture conditions do not always meet the plant cells requirements (Laimer 2003). Attempts to improve grapevines by genetic engineering techniques depend on the availability of reliable protocols for transformation, selection and regeneration. Major limitations were attributed either to the high degree of kanamycin sensitivity exhibited by grape (Colby and Meredith 1990), to strong genotypic differences affecting the regeneration capacity, or to the fact, that in particular in leaf and petiole explants, cells that are competent to regenerate new shoots may not be the same as cells transformed by agrobacteria.

Adventitious shoots from 'French Colombard' and 'Thompson Seedless' petioles originated from the epidermal and subepidermal layers, while transformation occurred at the cut surface and in internal tissues (Colby *et al*. 1991).

The choice of the best explant is a crucial decision, even today with many developed protocols. Leaf discs, cotyledons and stem cuttings represent complex explants which allow to regenerate plantlets with some success from many cultivars, including woody species (Laimer *et al*. 2005). Regeneration from petioli of *Vitis* rather seemed to give rise to chimeric regenerants, due to the fact that subepidermal and epidermal cells jointly contributed to an initiating promeristem (Colby *et al*. 1991).

Regeneration of grapevines is also feasible from embryogenic cultures (Kikkert *et al*. 1996; Perl *et al*. 1996; Gölles *et al*. 2000; Kikkert *et al*. 2000; Martinelli and Gribaudo 2001; Iooco *et al*. 2001). Furthermore regeneration of plants from single cells can be consider of major advantage for *Agrobacterium tumefaciens*-mediated gene transfer to achieve homogeneously transformed plants (Polito *et al*. 1989). Currently somatic embryogenesis appears to be the most promising approach to introduce new genes in woody crop species (da Câmara Machado *et al*. 1995).

Mezzetti *et al.* (2002) described a method based on the formation of meristematic bulk (MB) tissue with a high regenerative capacity, using adventitious shoots of two table grape cultivars 'Silcora' and 'Thompson Seedless' as a starting material. MBs were used to introduce the ovule-specific regulatory regions from *DefH9* of *Antirrhinum majus* and the *iaaM* coding region from *Pseudomonas savastanoi*, *DefH9-iaaM* (Koncz and Schell 1986; Rotino *et al*. 1997) into two grapevine cultivars, 'Silcora' and 'Thompson Seedless'.

Despite significant progress in genetic engineering, some cultivars of *V. vinifera* are recalcitrant to transformation. The supervirulent EHA105 *A. tumefaciens* and the wide host range A4 *A. rhizogenes* strains showed increased transformation efficiency compared to the widely used LBA4404 *A. tumefaciens* strain or the limited host range K252 *A. vitis* strain (Torregrosa *et al*. 2002).

The selection system for the recovery of transgenic fruit tree plantlets is a further crucial step. In poorly regenerating explants also transformed cells may die because of the isolation effect, if confronted with a high selection pressure from the beginning (Laimer 2003). Among the most commonly used selection genes are neomycin phosphotransferase (*npt*II), conferring resistance to aminoglycoside antibiotics, and phosphinothrycin acetyl transferase (*pat*), conferring resistance to the herbicide phosphinotrycin (Miki and McHugh 2004). Kanamycin selection, widely used in screening for transformants, is known to have inhibitory effects on the regeneration capacity of somatic embryogenic cultures and leaf disc of grapevine (Colby and Meredith 1990; Gölles *et al*. 2000). Wang *et al*. (2005) compared the effect of various concentrations of kanamycin and paromomycin on embryogenic cell suspension viability, transformation efficiency, and transgenic plant regeneration. Paromomycin (10-25 mg/l) induced an earlier killing effect on cell suspensions than kanamycin (40-100 mg/l). Transformation efficiency and the number of embryos developed on selection medium were positively correlated with an increase in paromomycin concentrations from 10-20/25 mg/l. Paromomycin was more effective than kanamycin in selection of transformed cells and induction of embryo development during selection. More recently positive selection strategies have emerged, like the use of transgenes able to utilize unusual carbon sources like xylose and mannose (Haldrup *et al*. 1998; Zhang *et al*. 2000), or encoding enzymes involved in hormone biosynthesis (Ebinuma *et al*. 1997; Kunkel *et al*. 1999; Ebinuma *et al*. 2001). Historically one of the first selection systems was herbicide resistance. In regions that traditionally support cereal crops, penetration of the viticulture industry has been difficult because of widespread use of 2,4-dichlorophenoxyacetic acid (2,4-D) broadleaf-weed killer on large industrial fields. Typical formulations of the ubiquitous herbicide are highly volatile and are prone to drifting long distances. Grapes display a high sensitivity to even minute exposure to 2,4-D, which causes serious injury, crop losses and even vine death. By incorporating the modified *tfd*A gene, which encodes for an enzyme that degrades 2,4-D to dichlorophenol (DCP), genetically modified grapevines cv. 'Chancellor' could tolerate up to twenty times the rate of 2,4-D used to control broadleaf weeds (Skirvin 2007).

The efficiency of phosphomannose-isomerase (PMI) and phosphinotricin acetyl transferase (PAT) as selectable marker systems to regenerate genetically modified grapevines (*Vitis* spp.) were investigated in *V. vinifera* L. cv 'Merlot', *Vitis* sp. 'Seyval blanc' and different rootstocks (*V. berlandieri* × *V. riparia*) (Reustle *et al*. 2003). Mannose was found to have only a minor selective effect on embryogenic tissue of grapevine. Grapevine embryogenic tissue was highly sensitive to phosphinotricin (PPT) treatments higher than 2.5 to 5.0 mg/L. Reustle *et al*. (2003) concluded that both selectable marker systems need further optimisa-

tion before being used as efficient selection and regeneration systems for genetically modified grapevines.

Transgenic approaches to virus resistance

Engineered protection offers a new approach to manage virus diseases allowing completely new avenues of protection. Strategies for genetic engineering of resistance to virus are based on three types of transgenes: a) plant-derived transgenes including pathogenesis-related (PR) and resistance (*R*) genes (Fermin-Muñoz *et al*. 2000), b) non-plant–, non-pathogen–derived transgenes, e.g. antibodies and antiviral proteins (Schillberg *et al*. 2001) and c) pathogen-derived transgenes (Sanford and Johnston 1985).

The use of plant-derived transgenes allowing the introduction of natural *R* genes from one plant species to another has obvious advantages, since in the public perception it is more readily accepted than genes from other organisms. Improvements in the identification and characterisation of such genes will enhance the development of this approach (Deng 2006).

The expression of the viral coat protein gene in transgenic plants induced similar protective effects as classical cross protection and was therefore distinguished as "coat protein-mediated" protection (Beachy *et al*. 1990). Since viral sequences encoding structural and nonstructural proteins were shown to confer resistance, this concept was enlarged and termed pathogen-derived resistance (PDR) (Lomonossoff 1995).

In the case of transgenic grapevines initially the use of translatable and non-translatable coat protein sequences yielded both immunity and recovery resistance in model plants, however both the number of protected lines as well as the level of protection against homologous virus strains seemed worthwhile improving. A further driving force for the modification of constructs were safety considerations concerning a) selection of viral sequences reducing the potential risk of recombination or b) mutations of the coat protein (*cp*) gene suppressing particle assembly, heterologous encapsidation and complementation (Balázs and Tepfer 1997).

One of the most damaging and widespread viral diseases affecting grapevine (Andret-Link *et al*. 2004) is caused by *Grapevine fanleaf virus* (GFLV), together with *Arabis mosaic virus* (ArMV) and other nepoviruses (Bovey and Martelli 1986). GFLV is spread both via propagating material and the specific nematode vector *Xiphinema index*.

The rugose wood complex of grapevine is found in most viticultural countries all over the world. The mealybug-transmitted vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) are involved in the aetiology of Kober stem grooving and corky bark, respectively, two of the syndromes of the complex (Minafra *et al*. 1997). Again, no natural resistance to these viruses is known in *Vitis* sp.

Coat protein genes of grapevine viruses, including *Grapevine fanleaf virus*, *Grapevine Virus A*, *Grapevine Virus B*, *Grapevine chrome mosaic virus* and Tomato ringspot virus have been employed as transgenes in different cultivars (**Table 2**). Different reports described the regeneration of transgenic grapevine plants expressing a chimeric GFLV *CP* gene after transformation of somatic embryos of the rootstock cultivars '110 Richter' (Krastanova *et al*. 1995; Gölles *et al*. 1998; Xue *et al*. 1999; Gölles *et al*. 2000), '41B' and 'SO4' (Mauro *et al*. 1995, 2000), and the *Vitis vinifera* cultivars 'Chardonnay' (Mauro *et al*. 1995), and self-pollinated 'Russalka' (Gölles *et al*. 1998, 2000). Gambino *et al*. (2005) and Maghuly *et al*. (2006) reported the regeneration and molecular characterization of transgenic grapevine plants of several cultivars transformed with the GFLV-CP gene in sense, translatable orientation or in

Table 2 Survey of genetic modifications of grapevine cultivars and rootstocks.

Species	Transformation	Trait	Genes	Explant	Reference
Vitis vinifera SO4 (*V. berlandieri* x *V. riparia*) and 41B (*V.v* x *V. berl.*)	*A. tumefaciens* LBA 4404	VR	GFLV cp gene	Anther-derived embryogenic callus	Mauro *et al*. 1995, 2000
110 Richter (*V. berlandieri* x *V. rupestris*)	*A. tumefaciens* LBA 4404	VR	GCMV cp gene	Anther-derived embryogenic callus	le Gall *et al*. 1994
110 Richter and *V. rupestris*	*A. tumefaciens* LBA 4404	VR	GFLV cp gene	Hypocotyl and anther-derived embryogenic callus	Krastanova *et al*. 1995, Laimer *et al*. unpubl.
110 Richter and *V. vinifera*	*A. tumefaciens* LBA 4404	VR	Cps of GFLV, ArMV, GVA and GVB	Immature embryo and anther-derived embryogenic callus	Gölles *et al*. 1998, 2000
V. rupestris	*A. tumefaciens*	VR	CP of ArMV	Somatic embryos	Spielmann *et. al*. 2000a,b
41B (*V.vinifera* x *V. berlandieri*)	*A. tumefaciens*	VR	Cp of GVA	Somatic embryos	Radian-Sade *et al*. 2000
V. vinifera 'Blaufränkisch', 'Nebbiolo', 'Lumassina'	*A. tumefaciens* LBA 4404	VR	GFLV Cp gene in sense or antisense orientation	Somatic embryos	Gambino *et al*. 2005
Rootstock 'RPG1'	*A. tumefaciens* LBA 4404	VR	GFLV Cp gene in sense or antisense orientation	Somatic embryos	Laimer *et al*. unpubl.
V. vinifera 'NeoMuscat'	*A. tumefaciens*	FR	Rice chitinase (RCC2)	Somatic embryos	Yamamoto *et al*. 2000
V. vinifera 'Merlot', 'Chardonnay'	Particle bombardment	FR	Chitinase ThEn42	Somatic embryos	Kikkert *et al*. 2000
V. vinifera 'Thompson Seedless', 'Chardonnay'	*A. tumefaciens*	FR, BR	PGIP	Somatic embryos	Aguero *et al*. 2005
V. vinifera 'Chardonnay'	Particle bombardment	FR, BR	Mag2 and MSI99	Somatic embryos	Vidal *et al*. 2006
V. vinifera 'Thompson Seedless'	Particle bombardment	BR	Shiva-1	Somatic embryos	Scorza *et al*. 1996
'110 Richter'	*A. tumefaciens*	BR	Truncated VirE2	Somatic embryos	Holden *et al*. 2003
V. vinifera 'Cabernet Franc'	*A. tumefaciens*	CR	SOD from Arabidopsis	Vegetative buds	Rojas *et al*. 1996
V. vinifera 'Silcora', 'Thompson Seedless'	*A. tumefaciens*	MFT	DefH9/iaaM	Meristemtic bulks	Mezzetti *et al*. 2002
V. vinifera	*A. tumefaciens*	MFT	PPO from grapevine in antisense orientation	Somatic embryos	Thomas *et al*. 2001
Vitis vinifera	*A. tumefaciens*	MFT	UDP:flavonoid 3-O-glucosyltransferase (UFGT)	Somatic embryos	Thomas *et al*. 2001

BR, bacterial resistance; CR, cold resistance; FR, fungal resistance; MTF, modified fruit traits; VR, virus resistance

antisense orientation (**Table 2**).

Resistance to GFLV in transgenic rootstocks expressing the GFLV CP gene has been recently reported after a three-year trial in a naturally infected vineyard in France suggesting that transgenic grapevines are likely to be of practical interest for the control of GFLV (Vigne *et al*. 2004). The study further indicated that transgenic grapevines did not favor the development of GFLV recombinant isolates to a detectable level (Vigne *et al*. 2004). Thus, GFLV-resistant transgenic grapevines could allow sustainable production while preserving the environment.

Pathogen-mediated resistance meanwhile has been shown to be RNA-mediated and based on a mechanism of co-suppression and post-transcriptional gene silencing (PGTS) or homology related gene silencing (Dougherty and Parks 1995; Wassenegger and Pélissier 1998). Sequence-specific RNA silencing processes in plants point to the existence of a natural defence mechanism of adaptive protection against viruses (Waterhouse *et al*. 2001; Voinnet 2001; Baulcombe 2004). Furthermore, many plant viruses encode proteins suppressing PGTS, suggesting a co-evolution of defence and counter-defence between the host and the invading virus (Voinnet *et al*. 1999; Wang *et al*. 2006).

The development of constructs containing inverted repeats of viral coat protein or replicase genes has been the last step to increase protection efficiency. PGTS has been achieved with high efficiency in transgenic plants expressing self-complementary hairpin RNAs (Smith *et al*. 2000; Wesley *et al*. 2001).

Transgenic approaches to fungal resistance

Botrytis cinerea, the causal agent of grey mould or botrytis bunch rot in grapes, downy mildew, caused by *Plasmopara viticola* (Berk. & M.A. Curtis), powdery mildew, caused by *Uncinula necato*r (Schwein.) Burrill (anamorph: *Oidium tuckeri* Berk), Eutypa dieback, caused by the ascomycete fungus *Eutypa lata*, seriously affect grapevines worldwide, particularly *V. vinifera* cultivars.

Polygalacturonases (PGs) are among the first enzymes secreted by a number of fungal and bacterial pathogens contributing to the aggressive decomposition of susceptible plant tissues (de Lorenzo *et al*. 2001). Fungal pathogens such as *Botrytis cinerea*, are all dependent on PGs to maintain full virulence on their respective hosts, and *B. cinerea* was recently shown to have at least six PGs, differentially regulated during the infection process and contributing to virulence and symptom development (Wubben *et al*. 2000; Kars *et al*. 2005). Polygalacturonase-inhibiting proteins (PGIPs) are plant cell wall proteins with a role(s) in plant defence, most notably their interaction with and inhibition of pathogens polygalacturonases (PGs) (Cook *et al*. 1999; de Lorenzo *et al*. 2001; Gomathi *et al*. 2006).

The importance of PGIPs in defence against fungal pathogens has been further demonstrated by the over-expression of various PGIP-encoding genes in native as well as heterologous hosts, e.g. a grapevine PGIP encoding gene, *Vvpgip1*, and PGIP purification from grapevine berries yielded a protein with strong inhibition activity against a crude extract of PGs from *B. cinerea* (de Ascensão 2001). *Vvpgip1* over-expressed in tobacco showed reduced *B. cinerea* symptom development. VvPGIP1 purified from transgenic tobacco and used to evaluate its interaction with and inhibition of individual PGs from *Aspergillus niger and B. cinerea* (Joubert *et al*. 2006).

V. vinifera cvs. 'Thompson Seedless' and 'Chardonnay' were transformed to express pear fruit PGIP-encoding gene (pPGIP) under the control of the CaMV 35S promoter (Aguero *et al*. 2005). Leaves of transgenic plants infected with *Botrytis cinerea* had reduced rates of lesion expansion. The development of Pierce's disease (PD) was delayed in some transgenic lines with increased pPGIP activity. PD-tolerant transgenic lines had reduced leaf scorching, lower *Xylella* titres and better re-growth after pruning than the untransformed controls (Aguero *et al*. 2005).

Genes encoding hydrolytic enzymes such as chitinase, which degrade fungal cell wall components, are attractive candidates for improving disease resistance. Transgenic rice expressing rice endochitinase exhibited resistance to blast (Nishizawa *et al*. 1999), while transgenic strawberry (Asao *et al*. 1997) harboring a rice chitinase possessed increased resistance to various fungal diseases, e.g. to powdery mildew *Sphaerotheca humuli*. The rice chitinase gene (RCC2), classified as class I chitinase, was introduced into the somatic embryos of grapevine (*V. vinifera* L. cv. 'Neo Muscat') by *Agrobacterium*-mediated transformation. The resulting transformants showed enhanced disease resistance to powdery mildew and anthracnose (Yamamoto *et al*. 2000).

In fighting Eutypa dieback, initial steps involve the understanding of the basic mechanisms involved. The fungus synthesizes the toxin eutypine, which is transported in the xylem and metabolized into eutypinol, which is not toxic for grapevines. A relationship was found between tolerance to this disease is related to the capacity of cells to convert eutypine to the corresponding alcohol (Roustan *et al*. 2000). Eutypine reductase from *Vigna radiata* (Vr-ERE, eutypine reducing enzyme) cDNA encoding an NADPH-dependent reductase with high affinity towards eutypine, confers resistance to the toxin in transgenic grapevine cells, a discovery opening new biotechnological approaches for the generation of grapevines resistant to *Eutypa* (Roustan *et al*. 2000).

Transgenic approaches to bacterial resistance

Grapevines are affected by bacterial diseases like crown gall (*A. tumefaciens* (Smith & Townsend) Conn) and *Xylella fastidiosa*, a xylem-limited bacterium causing PD.

A. tumefaciens causes biovar 3, is the predominant type isolated from grape plants (*Vitis vinifera* L.), has not been isolated from other plants and was renamed *A. vitis* (Ophel and Kerr 1990). *Agrobacterium* survives systemically in grape plants and incites a decay of grape roots, which is associated with the production of a chromosomally-encoded PG. PG has not been detected for other biovars and is associated with the ability of biovar 3 to cause a decay of grape roots (McGuire *et al*. 1991).

VirE2 is known to be required for optimal T-DNA transfer. The VirE2 protein is probably transferred to the plant cell independently of the T-DNA, where it binds to single-stranded T-DNA and subsequently plays a role in the import of the T-DNA to the plant nucleus. Embryo cultures of 'Richter 110' transformed with truncated *virE2* genes (lacking a region predicted to be associated with DNA binding) isolated from *A. tumefaciens* strains C58 and A6 and from *A. vitis* strain, CG450 expressed reduced susceptibility to crown gall (Holden *et al*. 2003). Two of the lines were resistant to infection by all three of the *Agrobacterium* strains. The effectiveness of such an approach under field conditions has not been proven.

PD induces symptoms as yellowing and gradual necrosis of leaves, uneven cork development and presence of petioles attached to the cane after leaf fall. The disease progresses rapidly, resulting in occlusion of xylem vessels and consequent water stress. Vine death often occurs within 2 years (Goodwin *et al*. 1988). Being vectored by several leafhoppers, which are difficult to control, conferring resistance to the grapevines appears a valid alternative, comparable to the situation met with grapevine viruses. *Xylella fastidiosa* contains a putative intact PG gene (van Sluys *et al*. 2003), which may contribute to bacterium virulence and systemic colonization of the host by degrading the pectin-containing pit membranes that separate adjacent vessels, releasing nutrients for the pathogen, triggering host vessel blockage and/or aiding in initial invasion (Harakava *et al*. 2001). Therefore PGIP expression could confer tolerance against this bacterium as well as against the fungal pathogen *Botrytis cinerea*, which indeed was observed on transgenic 'Thompson Seedless' and 'Chardonnay' (Aguero *et*

al. 2005).

Different strategies and genes have been used in genetic engineering to enhance resistance to major plants pathogens, including the use of antimicrobial peptides (AMPs) (Vidal *et al*. 2006). These are natural defensive compounds found in many organisms, ranging from bacteria to humans and plants, that protect the host from invading pathogens. Among these compounds are the magainins, isolated from the skin of the African clawed frog, *Xenopus laevis*. Magainins have a broad-spectrum antimicrobial activity inhibiting the growth of bacteria and fungi including major grapevine pathogens such as *A. tumefaciens* (Li and Gray 2001). Transgenic grapevines 'Chardonnay' (*V. vinifera*) carrying either a natural magainin-2 (mag2) or a synthetic derivative (MSI99) gene under control of the Arabidopsis ubiquitin-3 promoter showed some lines with enhanced resistance to crown gall and powdery mildew diseases in greenhouse tests (Vidal *et al*. 2006). 'Thompson Seedless' grapes have been transformed with the lytic peptide gene, Shiva-1 with the aim of producing plants resistant to microbial infections (Scorza *et al.* 1995).

Transgenic approaches to modified fruit traits

Breeding for seedless cultivars, changes in enzymes causing browning of damaged plant tissues and colour development, e.g. polyphenol oxidase (PPO) gene from grapevine in antisense orientation, with the aim of reducing PPO levels in the plant and UDP:flavonoid 3-*O*-glucosyltransferase (UFGT) involved in determining berry colour, arose recently from the rapid progress in grape genomics as potential novel targets (Thomas and Scott 2001). Extension of the growing range by increased cold resistance may further represent a further target for breeding (Colova-Tsolova *et al*. 2001). Rojas *et al*. (1996) engineered 'Cabernet Franc' with a superoxide dismutase gene from *Arabidopsis thaliana*, protecting grapevines from additional five degrees of killing frost that could make growing grapes in Canada more reliable.

Genes involved in the hormonal balance and transcription regulator gene from grapevine that controls flowering and fruit development might be used to increase yield. Mezzetti *et al.* (2002) transformed meristematic bulk (MB) tissue with ovule specific regulatory regions from *DefH9* of *Antirrhinum majus* and the *iaaM* coding region from *Pseudomonas savastanoi*, *DefH9-iaaM* (Koncz and Schell 1986; Rotino *et al*. 1997) conferring parthenocarpic fruit development into the genome of two table grape cvs. 'Silcora' and 'Thompson Seedless'. The transformed grape plants show normal vegetative growth and express the *DefH9-iaaM* gene in young flower buds (Mezzetti *et al*. 2002).

IMPROVEMENT OF NEW CONSTRUCT DESIGN

In order to produce resistant grapevines not only an efficient protection, but also environmental safety aspects were considered. To achieve social acceptance for genetically modified grapevines, possible risks must be limited by the use of appropriate constructs. This has been attempted by the construction of modified CP sequences (Gölles *et al*. 2000), e.g. truncated sequences, which are expected to produce smaller protein subunits possibly unable to self-assemble to empty viral capsids, as had been reported previously for native CP genes of ArMV (Bertioli *et al*. 1991), or to suppress protein translation by the use of antisense constructs. Recent advances in unravelling gene silencing and the synthesis of siRNA (small interfering RNA) should provide new tools for engineering stable and durable protection against viruses.

The constructs might be improved by avoiding backbone sequences unexpectedly integrated, particularly in the case of pBin19 derived plasmids (Schiemann *et al*. 2003), by the use of other reduced and optimized vectors, e.g. pGreen (Hellens *et al*. 2000).

A further advantage is represented by temporally and spatially inducible gene expression by the use of tissue specific promoters (Pühringer *et al*. 2000). A fruit- and ripening-specific proline-rich protein from grapevine (mrip1) was used to isolate a fruit- and ripening-specific promoter including a spectrum of hormone-, light-, phytochrome-, sugar- and stress-responsive elements (Burger 2006). In transgenic tobacco, the transcription was developmentally regulated and specific to the ovary and nectary-tissue specific of the developing flower. While low in immature flowers, expression rapidly increased to high levels visualized in the flower in full-bloom, followed by a decrease in the final stages of ovary development, providing a valuable tool for the genetic manipulation of fruit ripening in grapevine (Burger 2006).

To improve the understanding of structural and molecular requirements for seed specific gene expression in grape (*Vitis vinifera* L.), a 2S albumin gene *VvAlbl* from different cultivars of grape was chosen and seed-specific activity of the *VvAlb1* gene promoter was analysed directly from genomic DNA by using an improved version of the thermal asymmetric interlaced PCR (TAIL-PCR) procedure (Li *et al.* 2005).

The pathogen-inducible Mal d 1 promoter (Pühringer *et al*. 2000) might be an alternative to express transgenes in grapevine. In a first approach the inducibility of the *uid*A gene by *Plum pox virus* in *Nicotiana benthamiana* was analysed. Also herbaceous model plants carrying non-translatable versions of the cp gene of a non-aphid transmissible strain (PPV-NAT) under the control of the Mal d 1 promoter were tested successfully (Mendonça 2005).

Novel bi-directional duplex promoters (BDDP) constructed by placing two identical core promoters divergently on both upstream and downstream sides of their duplicated enhancer elements were shown to increase significantly the expression levels of marker genes (Li *et al*. 2004). Possibly, BDDP offer certain structural and functional advantages, including providing compact DNA sequence organization; enhancing communication and interplay between enhancer and promoter sequences and transcriptional factors; and increasing efficacy of transcription regulation and gene expression.

SAFETY ASPECTS AND PUBLIC PERCEPTION

Many concerns have been raised regarding potential ecological risks of transgenic plants. Although these concerns deserve attentive observation (Tepfer 2002), only experimental data in a step-by-step approach will allow a correct judgement on the value of these crops.

ARMGs conferring resistance to antibiotics have been discussed as potential sources of haszard. The *npt*II gene, conferring kanamycin resistance for selection of transformed plant cells, has been cleared by EFSA (2004), since it has a 13-year history of safe use in food crops and resistance to this group of antibiotics is widespread in naturally occurring microbes in humans and the environment. The Panel is of the opinion that with regard to safety there is no rationale for inhibiting or restricting the use of genes in this category, either for field experimentation or for the purpose of placing on the market. On the other hand, transgenic plants carrying a tetracycline resistance gene, under the current legal situation are to be excluded from applications for commercial release from the year 2008 on (EC 2001).

So far a number of genetically modified (GM) grapes have been created and tested in numerous field trials in the US (http://www.isb.vt.edu/cfdocs/fieldtests3.cfm), Europe (http://www.isb.vt.edu/cfdocs/globalfieldtests.cfm) and Australia (http://www.isb.vt.edu/cfdocs/globalfieldtests.cfm). For comparison it seems worthwhile to mention, that there were 25 field test releases of GM yeast in the USA between 1999 and 2005. There have not yet been commercial releases of GM grapes.

Social and ethical concerns have been expressed on the use of transgenic grapevines, sometimes creating a strong

climate of opposition. In France, the controversial acceptance and general confusion on the usefulness of GFLV-resistant transgenic grapevines prompted the Director of INRA to take a novel and unique initiative in 2001 (http:www.inra.fr/Internet/directions/SED/science-gouvernance/ITA-Vignes/index.html). A risk assessment study performed in the field with transgenic grapevines suggests no detectable environmental impact beyond natural background events regarding the emergence of recombinant GFLV species (Fuchs 2003). Whether virus-resistant transgenic grapevines will be made available to growers within a reasonable period of time depends on education, dialogue, and promotion of informed choices (Fuchs 2003). The severe detrimental impact of viruses, the strong demand for a reduction in the reliance on toxic agrochemicals for virus vector control, the pledge for a safe and sustainable viticulture, and the success of biotechnologies at offering alternatives to current control strategies, open the opportunities for practical use of virus-resistant transgenic grapevines.

ACKNOWLEDGEMENTS

The financial support by the BMLFUW and the EU-Project Resistvir is kindly acknowledged.

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