Resistance to viruses, phytoplasmas and their vectors in grapevine: a review

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ABSTRACT

The control of grapevine virus and phytoplasma diseases is currently based on prophylactic measures and cultural practices. Certification programs aim to avoid the introduction of diseased grapevines in healthy vineyards, and cultural practices aim to reduce the populations of virus vectors and to limit the spread of the virus. These approaches however are of limited efficiency. Additionally, the search for natural resistance against pathogens in grapevine has not led to any result. The implementation of genetic engineering provides new approaches to develop pathogen-resistant grapevines. Transgenic grapevines expressing virus resistance genes have been already obtained in several laboratories. The introduction of coat protein genes has been the most commonly adopted strategy. More recently, new approaches based on gene silencing, specifically triggering the plant defense mechanisms, have opened new ways for the engineering of pathogen-resistant grapevines. The possibilities, benefits and advantages, but also the risks involved with the introduction of transgenic crops in the fields, as well as their acceptance in the population, are discussed in this review.

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the oldest cultivated crops. In 2001, grapevines were grown worldwide on 7.8 million hectares, of which 62.7% in Europe (Office International de la Vigne et du Vin, 2002). However, grapevines, like any other plants, are exposed to many biotic stresses, e.g. insects, fungi, bacteria, phytoplasma, viruses, which are responsible for dramatic economic losses throughout the world, and which hamper the success of this crop. Grapevine is one of the woody plants most infected with viruses, with at least 55 species belonging to 20 different genera being recorded (Martelli, 2003). However, among all these virus diseases, the most important are the grapevine fanleaf disease, the leafroll disease, and the rugose wood

complex. For phytoplasma on the other hand, the Grapevine yellows (GY) disease, caused by genetically different phytoplasma, is the most important disease of grapevine and is distributed worldwide. Among them, Flavescence dorée (FD) phytoplasm is the most severe pathogen.

Virus-derived resistance remains the most promising strategy to engineer virus resistance in grapevine as an alternative to the use of agrochemicals and when the other ways drive to dead ends. Recently, it has been shown that expression of viral genes in plants can confer resistance to the challenging viruses, mainly by a mechanism called post-transcriptional gene silencing (PTGS) or RNA silencing (Lindbo et al. 1993, Baulcombe, 1996, Prins, 2003). PTGS is a general antiviral defence system in plants that is activated as a response to aberrant RNA (abRNA) or double-stranded RNA (dsRNA) accumulating during virus replication. According to this process, gene silencing would be activated naturally in virus-infected plants, and artificially in transgenic plants, when the transgene or its RNA is perceived as part of a virus (Ruiz et al., 1998). However, many viruses encode proteins that are able to suppress the plant RNA silencing response (for a recent review, see Voinnet, 2005).

Thus, using this increasing knowledge, development of new approaches for plant protection against virus diseases based on PTGS have been lately developed (Tenllado et al., 2004). Viral genes are engineered as sense-translatable, antisense, untranslatable or hairpin sequences. The resistance is increased by introducing inverted repeat transgenes, resulting in highly structured RNA transcripts (Smith et al., 2000). This strategy is of great interest for transformation/regeneration-recalcitrant crops like grapevine, because it allows the introduction of a limited number of genes while maintaining its phenotypical character. Furthermore, the use of hairpin transgenes has shown that only a few successful transformation events are needed for the generation of efficient resistance (Bucher et al., 2004).

In this review, initiated in the frame of the co-ordination of research on genetic resistance to plant pathogenic viruses and their vectors in European crops (ResistVir project) we present the efforts for resistance breeding employing natural resistance, when available, to pathogens and their vectors, as well as genetic engineering towards grapevine resistance against viruses and phytoplasma. Considering the public concerns expressed particularly in Europe about the release of genetically modified organisms in the fields, we also address the benefits and risks, environmental safety aspects, and social acceptance for genetically modified grapevines.

I. Presentation of the pathosystems

The grapevine fanleaf disease

Grapevine fanleaf disease is the major virus disease of grapevine worldwide (Bovey et al. 1990; Martelli and Savino 1990). Infected grapevines display malformation of the leaves, chlorotic mottling, yellow mosaic in addition to substancial crop losses, reduced fruit quality and shortened longevity. As an example, the fanleaf disease causes $ 1.5 billion annual losses to the French grapevine industry despite prophylactic measures and certification schemes for the nurseries (Fuchs 2006). Grapevine fanleaf disease affects 540 000 ha (60 % of the total acreage cultivated with grapevines in France) with highly detrimental effects in some vineyards, where more than 80% crop losses can be observed.

The aetiological agents of fanleaf disease, including infectious degeneration and grapevine decline, have been shown to belong to the *Nepovirus* genus in the *Comoviridae* family and to the unassigned *Sadwavirus* genus (Digiaro et al., 2007). They exhibit differential geographical distribution and have been split between the European, North African and Mediteranean basin (Turkey) and North American nepoviruses. Grapevine fanleaf virus (GFLV), the main causal

agent of grapevine fanleaf disease, is ubiquitous, conversely to other nepoviruses displaying a restricted geographical distribution (Table 1). An extensive review describes the main features of the biological, serological and molecular properties of GFLV (Andret-Link et al. 2004). It has been shown that multiple infections by divergent GFLV isolates can occur in a single vine (Vigne et al. 2004b) as well as mixed-infections with other nepoviruses and viruses from different genera.

Nepoviruses are mainly transmitted in a semi-persistent manner by ectoparasite longidorid nematodes (Andret-Link and Fuchs 2005). Viruses can also be disseminated by human activities such as grafting and soil transfer, by seeds of some herbaceous hosts and suspectedly by pollen.

Despite the global implementation of the sanitary selection and certification schemes for mother plants of rootstocks and *V. vinifera* varieties, the use of culture practices (fallows over ten years), soil desinfection and nematicides with the use of environmental unfriendly agrochemicals (some of them being already prohibited in the EU like aldicarb), fanleaf disease remains an expanding pandemic in vineyards worldwide and is still a major threat to the grapevine industry (Andret-Link et al. 2004, Esmenjaud et al, 2005).

The grapevine leafroll-associated virus complex

Grapevine leafroll is probably the most widespread virus disease of grapevine (Martelli and Boudon-Padieu, 2006). Affected vines display yellowing (white varieties) or reddening (red varieties) of limbs, with veins remaining green, as well as downward rolling of the leaf borders. Moreover, grapevine leafroll disease affects the phloem anatomy, delays the ripening of bunches and decreases vigour and yield (by 15-20% in average). No single virus species has been determined as the causal agent of the grapevine leafroll disease, but a range of distinct species belonging to the *Closteroviridae* family have been associated to leafroll

disease. These filamentous and phloem-limited viruses are designated as 'Grapevine leafroll-associated virus' (GLRaV) followed by a specific number, from GLRaV-1 to -9, and classified in the genera *Ampelovirus*, *Closterovirus* and, tentatively, *Crinivirus* (Table 2). GLRaV-2 is also involved in graft incompatibility.

Like the majority of viral and prokaryotic pathogens of grapevine, leafroll viruses are transmitted through vegetative propagation and grafting, a situation that implies to implement stringent sanitary controls, whenever possible, during the process of producing plants. Thermotherapy and/or meristem culture, as well as antiviral compounds, have been used to sanitize certain varieties from leafroll viruses (Komar et al., 2007, Panattoni et al., 2007).

In addition to vegetative propagation and grafting, the *Ampelovirus* species GLRaV-1, -3, -5 and -9 are also vectored experimentally and naturally by several species of Homopterans belonging to families *Pseudococcidae* (mealybugs) (*Heliococcus bohemicus*, *Phenacoccus aceris*, *Planococcus* spp., *Pseudococcus* spp.) and *Coccidae* (soft scales) (*Parthenolecanium corni*, *Pulvinaria vitis*, *Neopulvinaria innumerabilis*). The natural vectors, if any, of the other GLRaV species are unknown. The existence of several viral entities and, for some of them, of two modalities to spread out in vineyard render the epidemiology of leafroll disease a complex issue, that cannot be controlled by simple measures. To date, control of leafroll relies mainly on prophylactic controls of mother plants, by using detection methods with steadily better sensitivity, specificity and use facility. However, virus-free plants can be re-infected in the vineyard by natural vectors. Moreover, most rootstock varieties can be symptomless carriers of leafroll viruses and GLRaVs are extremely difficult to detect in rootstock material (Beuve et al., 2007) and many viral species are still insufficiently known at the molecular level. Chemical control of insect vectors can also been envisaged, however the lack of epidemiological knowledge on the precise vector activity of insects provides a poor basis for an optimal use of insecticides.

Flexivirids in grapevine

The filamentous viruses of the family *Flexiviridae* that infect grapevine (*Vitis* spp.) are phloem-limited viruses belonging to three different genera (Martelli *et al*., 2007). Grapevine viruses A, B and D are grouped in the genus *Vitivirus* (Martelli *et al*., 1997), while Grapevine Rupestris stem pitting-associated virus (GRSPaV) and Grapevine berry inner necrosis virus (GINV) belong to the genus *Foveavirus* (Martelli and Jelkmann, 1998) and *Trichovirus* (Yoshikawa et al., 1997), respectively. The first four viruses are associated to the Rugose Wood (RW) complex of the grapevine (Martelli, 1993). Recently, a report for a close association of GRSPaV to vein necrosis was given (Bouyahia et al., 2005). GINV is instead the agent of the homonymic disease in some Japanese varieties. GRSPaV is frequently detected in grapevine worldwide (Table 3), with 2 to 4 variants in the scion accessions and only one in rootstocks (Meng et al., 2006). Their latency condition in different *Vitis* species and hybrids is widely known, since symptom expression is often elicited when virus sources are grafted on healthy grapevine indicators (Savino et al., 1989). However, self-rooted vines in old viticultural areas (Martelli et al., 1994) or several table grape varieties may show stem grooving and corky alterations directly in field (Bonavia et al., 1996).

The vitiviruses (GVA, GVB and GVD) and the foveavirus (GRSPaV) infecting grapevines are closely associated to the RW disease. This complex disease can be sorted out, by graft transmission on specific indicators, in four different syndromes. Essentially, grooving and pitting on scion and/or rootstock, tickness above the bud union and the typical internodal swelling of the canes are the main symptoms, observed after months to years-long indexing trials (Martelli and Boudon-Padieu, 2006). Strict etiological relationships were described between GVA and the Kober stem grooving and GVB and corky bark, respectively (Chevalier et al., 1995, Bonavia et al., 1996). The involvement of some of GRSPaV strains in induction of Rupestris stem pitting or its association to the Shiraz decline is currently investigated

(Habili et al., 2006; Lima et al., 2006). Since co-infections of several of these viruses are frequent in grapevines, still more work is required to clarify their etiological role.

Several molecular variants were described for the vitiviruses (Sciancalepore et al, 2006, Murolo et al 2008, Goszczynski and Jooste, 2003; Shi et al., 2004) and GRSPaV (Meng et al., 1999; Nolasco et al., 2006), sometimes coinfecting the same grape accession. If, as for GRSPaV, a differential presence of variants could be stably associated to some *Vitis* hosts (Meng et al., 2006), the molecular variability found in GVA and GVB does not account for a sharp linkage to geographical origin or symptom expression.

Peculiar trait for the epidemiology of GVA and GVB is the grape-to-grape transmission by pseudococcid mealybugs and a scale insect (Martelli and Boudon-Padieu, 2006), whereas GRSPaV has no known vectors but is suspected to be pollen-borne and therefore transmitted at a low percentage by seeds. GINV is transmitted in vineyards by an eryophyid mite.

Grapevine phytoplasma diseases

Phytoplasma are wall-less pathogenic bacteria, belonging to the class *Mollicuta* and responsible for severe diseases in grapevine (Martelli and Boudon-Padieu, 2006). They are obligate intracellular parasites, restricted to phloem sieve elements of the infected plants, and transmitted by phloem-sucking leafhoppers and planthoppers. Phytoplasma are nonculturable *in vitro*, and their classification is mainly based on the study of the variability of 16S rRNA sequences. They have been included under the genus '*Candidatus* Phytoplasma' (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group, 2004), and a new complete re-classification has been proposed, based on the virtual restriction analysis strategy carried out on the phytoplasma 16S rDNA sequences deposited in GenBank (Wei et al., 2007). The molecular mechanisms responsible for the symptom expression in infected plants are only partially known (Jagouiex-Eveillard et al., 2001). Diseased plants show different

symptom patterns such as: abnormal proliferation, leaf chromatic alterations (yellows), flower modifications (virescence, phyllody), witches' broom, stunting.

Grapevine yellows (GY) is severe and worldwide distributed disease complex caused by genetically different phytoplasma. Infected plants of *V. vinifera* shows leaf rolling and curling, along with yellowing or reddening, rubbering of the canes and desiccated clusters. On the other hand, the epidemiology of the different GY diseases strictly depends on the phytoplasma involved and, in turn, by the specific vector transmission efficiency. Flavescence dorée (FD), the most severe disease within GYs, is caused by phytoplasma belonging to 16SrV-C and 16SrV-D taxonomic subgroups, with a proposed name of '*Candidatus* Phytoplasma vitis' (Firrao *et al.*, 2005), and specifically transmitted by the ampelophagous leafhopper *Scaphoideus titanus* Ball (Schvester *et al.*, 1961). Other types of GYs have been described in Europe, in Australia (Magarey and Wachtel, 1986) and in several American countries, and more than 6 genetically different phytoplasma have been found associated with these diseases (Table 4). Among them, Bois noir (BN), widely present in Europe, is caused by the phytoplasma belonging to 16SrXII-A subgroup, proposed as '*Candidatus* Phytoplasma solani' (Firrao et al., 2005) and transmitted by the polyphagous planthopper *Hyalesthes obsoletus* Signoret (Maixner, 1994, Sforza et al., 1998). FD and BN, were first reported in France (Caudwell, 1957; 1961) and are responsible for serious crop losses in many European countries (Boudon-Padieu, 2003).

II. Natural resistance breeding against pathogens and vectors

The search for natural resistance in plants against pathogens and their vectors has been a focus of attention among the scientific community for a long time (for a recent review of natural

resistance to plant viruses, see Maule et al., 2007 and references therein), however with different outcomes depending on the type of crop and pathogen.

Natural resistance to nepoviruses and their vectors

No native dominant resistance genes triggering an hypersensitive or extreme resistance against any viral disease have been found in grapevine towards GFLV or ArMV in wild or cultivated grapevines (Lahogue and Boulard 1996). Conventional breeding for virus-resistance using dominant genes through hybridization schemes cannot be developed. However, the strategy of exploiting recessive resistance genes (i.e. eukaryotic translation initiation factor eIF4E multigenic family) has not yet been explored and remains a promising idea (Ruffel et al. 2002).

High resistance to *Xiphinema index*, vectoring GFLV, has been discovered 25 years ago in *Muscadinia (Vitis) rotundifolia* (Bouquet, 1981; Staudt and Weischer, 1992). But *Muscadinia (Vitis) rotundifolia* is not suitable as rootstock because of its graft-incompatibility with *V. vinifera*, its poor rooting ability and its high susceptibility to lime-induced chlorosis. Nematode-tolerant grapevine hybrids were obtained but cannot totally impede nepoviruses to be transmitted to their rootlets and remained extremely susceptible to the virus itself. Implementing a tolerant or resistant program of grapevine toward longidorids may be useful, but definitely not sufficient against the fanleaf disease.

Natural resistance to leafroll viruses

To date, no natural source of resistance or tolerance to leafroll has ever been found in grapevine, neither in *V. vinifera* varieties nor in rootstocks (Martelli and Boudon-Padieu,

2006). The screening of a wide range (223 accessions) of American, Asian and Euro-Asian vine species, as well as of interspecific hybrids, by using green-grafting as the inoculation method, revealed no resistance to GLRaV-1 and -3 (Lahogue and Boulard, 1996). However, these authors conclude that the inoculation method they used is probably not the most suitable for such a screening, because the inoculation pressure is probably to high by grafting and does not allow to show possible resistance mechanisms occurring at early stages of infection, and also because some accessions, especially from genera other than *Vitis*, are graft-incompatible.

American and Euro-Asian rootstocks are usually symptomless carriers of leafroll viruses. However, observations of virus prevalence in vineyard trials lead Ioannou et al. (1997) to suggest that some rootstocks are less susceptible to GLRaV-3 and/or the vector than *V. vinifera* varieties.

Grapevine resistance to the vector and/or to the transmission process could also be interesting. However, to our knowledge, no such property has ever been reported. Resistance to scale insects is unlikely to exist in grapevine, because of their polyphagy; indeed the species feeding on grapevine also accept a broad range of other plants as hosts. In addition, the phloem-restriction of leafroll viruses and their acquisition and inoculation by phloemophagous vectors render unlikely any resistance to the transmission process. Furthermore, the search for vector resistance is rendered difficult by low transmission rates, as well as by our poor knowledge on the transmission mechanism (Cid et al., 2007). In herbaceous plants, there is at least one well-known gene (tomato *Mi-1*) inducing resistance to phloem-feeding invertebrates (Kaloshian and Walling, 2005; Maule et al., 2007), but its effect on scales has not been studied. More generally, there are only few reports of varietal resistance to scale insects in crops; e.g. in *Citrus* spp. (Franco et al., 2004; Boyero et al., 2007).

Natural resistance to *Flexiviridae*

Prevention of the grape-to-grape spread in nursery and vineyards and sanitation to produce virus-free grapes and rootstocks are still the only measures to control these viruses (Barlass, 1987; Wang et al., 2003). Anyway, if the meristem tip culture gave satisfactory results in removing vitiviruses from explants (Bottalico et al., 2000; Gambino et al., 2006), the somatic embryogenesis seems to be the most promising procedure for GRSPaV elimination, which is indeed recalcitrant to more traditional *in vitro* practices (Gribaudo et al., 2006). No resistance gene has been reported yet in *Vitis* germplasm for attempts of breeding against flexivirids infection. A co-evolution between viruses and host could be the reason of their large spread and infection success.

Natural resistance to phytoplasma

A strategy for controlling phytoplasma diseases is based on the selection of resistant, tolerant or not susceptible plant varieties. This selection can be carried out either by infection experiments on different varieties (Jarausch et al., 1999; Sinclair et al., 2000) or by molecular techniques, such as the detection of RAPD markers associated with the resistant varieties (Cardena et al., 2003). Unfortunately, resistance or tolerance to phytoplasma is not frequent in many host species, thus this strategy often results in a frustrating and useless work.

Up to now, none of the examined *Vitis* species have been found immune or resistant to the phytoplasma associated with GYs. Consequently, FD has been declared as a quarantine disease and chemical treatments against its vector *S. titanus* are mandatory. In fact, when the vector populations are not controlled, FD rapidly spreads in grape yards. In order to develop environmental friendly control methods for FD , biological control trials for *S. titanus* have been conducted by using: (i) natural antagonist insects such as *Lonchodryinus flavus* and

Gonatopus peculiaris, Hymenoptera (Drinides) with predator and parasitic activity (Malausa *et al.*, 2003); (ii) myco-insecticides such as *Beauveria bassiana* and *Poecylomyces fumorosesus*. These methods proved to be unsuccessful in field conditions because of the influence of environmental parameters on the biological activity against *S. titanus.*

Other FD control strategies involve agricultural practice. Several research articles report the efficacy of a careful winter pruning, or pollard, in reducing the effects of the disease. These studies were carried out on different cultivars, such as Chardonnay, Garganega, Perera, Prosecco, Barbera and Cabernet Sauvignon, giving encouraging, but often not reliable, results (Arnò *et al*., 1993; Osler *et al*., 1993). Spontaneous remission of symptoms (recovery) in FD affected vines has also been reported (Caudwell, 1961; Belli et al., 1978; Osler *et al*., 1993), often, but not always, accompanied by an absence of phytoplasma infection.

Overall, it appears that the search for natural resistance is such a time-consuming and uncertain prospect that the most favoured approach now relies on biotechnological methods aiming to obtain transgenic resistance against economically important pathogens.

III Transgenic resistance breeding

The genetic engineering technology has opened over the last decade new ways to introduce resistance against pathogens in all kind of plants (for a recent review, see Prins et al., 2008; Laimer, 2006, 2007). This technology is applicable to any plant susceptible to be transformed and regenerated.

Genetic engineering for Nepovirus resistance in grapevine

Several attempts were made to express viral genes in grapevine or herbaceous hosts that may lead to virus resistance. Several candidate genes for transgenic resistance were used : the coat protein (CP) genes of nepoviruses such as GFLV (Bardonnet et al., 1994; Gambino et al., 2005; Gribaudo et al., 2005; Maghuly et al., 2006; Mauro et al., 1995, Krastanova et al., 1995; Gölles et al., 2000; Valat et al., 2006, Xue et al, 1999), ArMV (Bertioli et al., 1991; Spielmann et al., 2000; Gölles et al., 2000), Grapevine chrome mosaic virus (Brault et al., 1993), Tomato ringspot virus (Yepes et al., 1996) and Tomato black ring virus (Pacot-Hiriart et al., 1999), other genes like the movement protein (MP) gene (Valat et al., 2006), the VPg (genome-linked viral protein) gene (Sun et al., 2001) or the RNA-dependent RNA polymerase. In addition, grapevine were transformed with conserved sequences of GFLV, ArMV and Raspberry ringspot virus in inverted repeat constructs (Reustle et al., 2005, 2006), in order to induce multiple virus resistance.

Phenotyping for resistance evaluation remains the time- and space-consuming bottleneck of the nepovirus-derived resistance strategy, since these viruses are naturally and obligatory transmitted through an ectoparasite longidorid. Many attempts have been made, including pre-screening approaches using a herbaceous host such as *Nicotiana benthamiana* to assess the performance of the constructs and showing in many cases immunity and recovery through a PTGS-induced defence mechanism in this herbaceous host (Jardak-Jamoussi et al., 2003, Reustle et al., 2006). Nevertheless, most of the time, this predicting phenotyping cannot be applied to grapevine since the expression of the resistance relies not only on the construct but also on the genomic location of insertion, as well as on the number of copies integrated.

Optimization for the evaluation of the transgenic grapevines has been attempted by graft inoculation (Barbier et al., 2000, Valat et al., 2003), micro-grafting (Reustle et al. 2006), agro-inoculation (Winterhagen et al., 2006), protoplast electroporation (Valat et al., 2006). For this

latter approach, inhibition of GFLV was observed in protoplasts of transgenic grapevine clones expressing CP or MP only, when protoplasts were electroporated with purified GFLV particles, but not with viral RNAs. Unfortunately, resistance of protoplasts of transgenic clones cannot be used to predict susceptibility to GFLV infection at the plant level (Valat et al., 2006).

The natural transmission remains the most reliable way to evaluate transgenic lines. Dual *in vitro* culture system of the viruliferous nematode and grapevine are being assessed (Reustle et al. 2006). To conclude about phenotyping for resistance, natural conditions of infection should be used in a first step in confined greenhouses, where the transgenic rootstocks are grown in nepovirus-longidorid infested soils. Then the evaluation could be made under field conditions, after extensive and pro-active communication on risks and benefits and general acceptance of such trials with growers, associations and public through debates and local steering commitees. An evaluation with five transgenic rootstock lines expressing the full-length translatable GFLV CP gene, in an open-field trial at INRA Colmar is presently under progress (O. Lemaire, personnel communication).

Transgenic resistance to leafroll disease

Leafroll viruses have not received yet so much attention for transgenic resistance engineering, probably because, apart from GLRaV-2, they have no herbaceous host plant on which preliminary experiments can be set up to challenge transgenically expressed viral genes. Moreover, leafroll ampeloviruses can imply transformation of either the rootstock, with the prospect to reduce the risk of man-driven spread through propagating material, or the variety, with the prospect to reduce the spread by natural vectors, or both the rootstock and the variety. Many attempts to produce leafroll-resistant transgenic grapevine lines targetting GLRaV-2 and -3 have been reported. They rely on approaches such as: (1) virus-derived

constructs from the CP gene (Ling et al., 1997a, 1997b; Xue et al., 1999; Krastanova et al., 2000; Ling et al., 2001; Burger et al., 2003) and from the MP genes (Freeborough and Burger, 2006), (2) ribosome-inactivating proteins from other plants (Burger and Wilsen, 2001), (3) recombinant antibodies (Fischer and Schillberg, 2003; Nölke et al., 2004; Cobanov et al., 2006). *N. benthamiana* plants transformed with the GLRaV-2 CP gene displayed resistance to the same virus, whereas homologous resistance is obtained in grapevines expressing the CP gene of GLRaV-3 (Gonsalves, 2000, 2001). GLRaV-2 resistance of *N. benthamiana* expressing the CP gene occurs through a PTGS mechanism (Ling et al., 2008). Transgenic tobacco plants with a mutated form of GLRaV-3 HSP70h gene (70-kDa heat shock protein homolog), a protein involved in viral movement and ATP hydrolysis, showed resistance to an unrelated virus, Potato virus X (PVX, *Flexiviridae*) (Freeborough and Burger, 2006). In addition to the CP and HSP70h genes, other viral genes can be considered with the aim of imparting pathogen-derived resistance. All known closterovirids possess two gene blocks (Dolja, 2006): one is 5'-located and codes for the replicase complex, the other is 3'-located and encodes products involved in virion assembly (CP and the components of the virion 'tail'; Alzhanova et al., 2007) and cell-to-cell and systemic movement. Whereas *Closterovirus* members possess 7 genes in the 3'-block, whose function has been elucidated for Beet yellows virus (BYV, type-member of the genus) and other species, the 3'-block of the few known *Ampelovirus* members displays up to 10 genes, some of which without known function so far. It is not excluded that one or more of the peptides expressed from this block may be involved in virus-plant or virus-vector interactions (Dolja, 2006). Interestingly, the most 3'-terminal gene of GLRaV-2 and of BYV encodes a suppressor of RNA silencing (Chiba et al., 2006), however the ortholog gene in ampeloviruses, if any, has not been identified yet. Knowledge of genomics of leafroll viruses and related species is expanding rapidly and will bring forth possible new candidates for obtaining pathogen-derived resistance.

Transgenic control of *Flexiviridae.*

Preliminary experiments for inducing virus resistance by *Agrobacterium*-mediated transformation with GVA and GVB were conducted on *Nicotiana* species. When transgenic lines of *N. benthamiana* (GVA) or *N. occidentalis* (GVB) transformed with the CP genes were tested, the correct translation products were detected in some lines. However, there was no correlation between tolerance to challenging virus and the amount of CP expressed, arguing for a leading effect of transgenic mRNA in controlling virus replication. In a few lines (R1 generation), the virus titer was reduced up to 10% and most of the plants were symptomless (Minafra et al., 1998). In Israel the GVA CP gene was similarly inserted in *N. benthamiana* (Radian-Sade et al., 2000). In some of the lines the resistance level (delayed or strongly reduced virus accumulation) was again not correlated with the presence of the transgenic virus protein. Heteroencapsidation between GVA and GVB (i.e. production of a chimaeric virus shell due to the assembly of both CPs) was demonstrated in transgenic and double infected *Nicotiana* plants, but this event did not modify at all the genetic structure of virus RNAs (Buzkan et al., 2001).

The effect of the sense and antisense RNAs of GVA and GVB MP genes was initially investigated in transgenic *Nicotiana* plants challenge-inoculated with homologous viruses. R1 seedlings of about half of the lines expressing antisense MP genes showed a significant reduction of virus titer, with ELISA readings lower than 30% than non-transgenic control, and did not show symptoms up to 18 days post-inoculation (dpi), whereas a moderate protection was observed in some lines expressing MP genes in sense orientation (Buzkan et al., 2000).

In grapevine, embryogenic tissues of *V. rupestris* and *V. vinifera* cv. 'Superior seedless' were transformed with the GVA or GVB MP genes either in sense or antisense orientation. Interestingly, the (+)-sense expressing plants showed abnormal growth and were difficult to

propagate *in vivo* for challenging tests (Martinelli et al., 2002). A successful transformation of the rootstock 41B with the GVA CP gene was also described (Radian-Sade et al., 2000), with some regenerated lines submitted to further testing. Transgenic grapevines containing GVA or GVB CP genes were obtained by transformation of embryogenic cultures of *V. vinifera* cv. 'Russalka' (Gölles et al., 2000). All transgenic grapevine tested were susceptible to homologous and heterologous virus infection, obtained either through grafting or by transmission with viruliferous mealybugs. Even if these lines accumulate transgenic mRNAs and proteins, the high titer of the transgenic products is therefore not correlated to virus resistance. The interspecific homology between CP genes of GVA and GVB (57%) may elicit, in double infected grapes as well as in infected transgenic plants, recombination events that could in principle lead to new chimaeric viruses. However, no recombination event was detected, and the genetic structure of the virus CP genes was not substantially modified in conventionally co-infected grapevines or when retrieved from CP-transgenic grapevines infected by the heterologous virus.

Transgenic control of phytoplasma

The impossibility of cultivating phytoplasma *in vitro* is a major obstacle to the development of efficient methods to control these pathogens. Additionally, the complete sequences of the genomes of only two phytoplasma strains (OY-M and AY-WB), belonging to the '*Candidatus* Phytoplasma asteris' species, have been determined (Oshima et al., 2004; Bai et al., 2006). From this information, it appears that the phytoplasma genome lacks many genes considered to be essential for the cell metabolism, such as the ATP synthase. On the other hand, membrane transporter genes (ABC-type transporters), for the uptake of nutrients from the host cell, have been found (Oshima et al., 2004).

Different genetic approaches, which enhance natural plant defence by promoting cell death at the site of infection via plant hypersensitive response (Belbahri et al., 2001), or inhibit pathogen growth by transgenic expression of anti-microbial peptides (Osusky et al., 2000) or single-chain variable-fragment antibodies (Le Gall et al. 1998) have been explored to generate resistance against pathogens. It was for example demonstrated that polyclonal, monoclonal and single-chain variable fragment (scFv) recombinant antibodies directed against spiralin, the immunodominant membrane protein of the culturable mollicute *Spiroplasma citri*, inhibit the *in vitro* growth of the pathogen (Malembic et al., 2002). In addition, a few recent reports suggested that anti-apoptotic genes of human, nematode, and baculovirus, once introduced into plants, may provide broad-spectrum resistance to fungal, bacterial and viral diseases, by blocking programmed cell death and preventing tissue necrosis (Dickman et al. 2001; Lincoln et al. 2002).

A plantibody-based approach to induce resistance against *Stolbur* phytoplasma ('*Ca.* Phytoplasma solani'), the aetiological agent of Bois noir disease in grapevine, was assessed in transgenic tobacco plants expressing a scFv antibody specific for the immunodominant membrane protein of *Stolbur* phytoplasma, and targeted in distinct plant compartments by using different promoters and different signal peptides. However, no significant resistance was observed when *Stolbur* phytoplasma was transmitted by grafting or by vectors to these transgenic tobacco plants (Le Gall et al., 1998; Malembic-Maher et al., 2005).

Transgenic *Paulownia* plants constitutively expressing the gene encoding the cecropin Shiva-1, were also assessed for resistance against the *Paulownia* witch's broom phytoplasma (taxonomic group 16SrI)(Du et al., 2005). Cecropins are peptides segregated from *Hyalophora cecropia* that have broad antibacterial activities. Previous studies have demonstrated that mycoplasma, which belong to *Mollicutes* class, are very sensitive to Shiva-

1 at low concentrations. Resistance to *Paulownia* witches' broom disease increased significantly in shiva-1-transgenic *Paulownia*, and Shiva-1 expression correlated with lower phytoplasma concentrations and less symptoms in infected transgenic *Paulownia*.

For the engineering of genetic resistance to phytoplasma in grapevine, it could be more advantageous to engineer phytoplasma-resistant rootstocks rather than individual grapevine varieties. Phytoplasma move to the root apparatus of vine plants during winter. If phytoplasma can be controlled during winter with genetically-engineered resistant rootstocks, chances of disease recurrence in the coming year would be significantly lower. Furthermore, the use of phloem-specific promoters, which are susceptible to direct specific delivery of anti-apoptotic or anti-microbial peptides to phloem sieve elements where the phytoplasma reside and multiply, could minimize the unnecessary exposure of non-target plant tissues to the anti-microbial agents. Recent experiments conducted with the promoter of the AtSUC2 gene (sucrose-H+ symporter) from *Arabidopsis thaliana* (Stadler and Sauer, 1996) for example showed a phloem-specific expression of a reporter gene in phloem companion cells of photosynthetic leaves (Zhao et al., 2004, Maghuly et al., 2008).

IV. Public acceptance and safetey aspects

In producing resistant grapevines not only an efficient protection, but also environmental safety aspects need to be considered. To achieve social acceptance for genetically modified grapevines, possible risks must be limited by the use of appropriate constructs. Many concerns have been raised regarding potential ecological risks of transgenic plants. Although these concerns deserve attentive observation, only experimental data in a step-by-step approach will allow a correct judgement on the value of these crops.

Perception and acceptance

Although the general public perceives it otherwise, work with GMPs is strictly regulated in most countries worldwide. The European Directives 90/219 and 90/220 regulate work with GMPs in contained systems and deliberate release. Modifications were added according to 98/81/EU, introducing issues of liability and public hearings, and regulating the position of public parties in cases of deliberate release. The directive modifying conditions for deliberate release and commercialisation 2001/18/EG of March 2001 was converted extremely slowly into national laws, leading even to legal consequences in the EU. However, the approval of field trials is being handled quite differently across Europe, which to a certain extent represents the most hesitant area worldwide in adopting the technology.

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). This initiative was based on a wide consultation and the promotion of pro-active and transparent dialogues with stakeholders (Fuchs, 2003). Thus, representatives of the scientific community, grape growers, nurseries, environmental protection agencies, and the public at large were invited to debate on the legitimacy and relevance of research activities on transgenic grapevines engineered for resistance to GFLV. This unique experience lasted almost for two years and called for a strong support of research in this controversial field. However, it did not consider favorably any commercial release of transgenic grapevines in the near future (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.

Environmental safety issues

Environmental safety issues have been expressed against the field release of transgenic plants, including grapevines. Such issues are particularly relevant in the case of a perennial crop species like grapevine, because it is grown for many years in the field, thus increasing the probability of occurrence of unintended phenomena (Laimer et al., 2005).

Possible interactions between products of the viral transgene, either RNA or protein, and an infecting virus, e.g. synergism, heteroencapsidation and recombination have been addressed as potential risks of the transgenic virus resistance approach (Tepfer, 1993, 2002; Robinson, 1996; Aaziz and Tepfer, 1999). Virologists do not consider heterologous encapsidation as a problem because the phenomenon is limited to a single transfer. The transcapsidated virus becomes defective with regards to the new host and should not be able to propagate without a helper virus. The assumption that transcapsidation may contribute to the introduction of a new virus into a new ecological niche triggered the formulation of safety recommendations: (a) not to express a coat protein in a plant that is not its natural host and (b) to create a biological containment system.

The formation of empty particles by a self-assembly process would be nothing new to transgenic plants, since empty particles are present also in purification of naturally occurring

infections, and can be separated as single fractions by conventional density gradient centrifugation procedures (Quacquarelli et al., 1976). In a preliminary study about the importance of truncated proteins in the process of protein folding and self-assembly, the truncated constructs - when transformed into *N. benthamiana* and *Vitis vinifera* - did not produce any VLPs (Castellano and Laimer, unpublished data, Gottschamel, 2008).

The occurrence of heterologous encapsidation was demonstrated in transgenic herbaceous host plants, *N. benthamiana* and *N. occidentalis* expressing the CP gene of GVA and GVB, respectively, which were challenged with the heterologous virus, as well as in co-infected nontransgenic *Nicotiana* (Buzkan et al., 2001). No information is available on such phenomena ocuring under conditions of natural virus infection in grapevines in the vineyard. 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).

One of the major problems of transgenic plants containing virus-derived genes is the possibility of recombination between viral transgene transcripts and RNAs from field viruses that infect transgenic plants. Such a recombination could allow the production of viruses that have the same properties that parental lineages (Vigne et al., 2005) or a new type of viruses having new biological properties such as increased pathogenicity, expanded host range, different specificity of vector-assisted transmission (Ding et al., 1996; Monci et al., 2002; Rubio et al., 1999). Interspecies recombination has been recently studied between a transgene from a resistant transgenic plant and unrelated viruses. Such hybrid plant viruses containing unrelated genes exhibited a selective disadvantage, first by being targeted by the silencing resistance mechanism and then not being competitive with the parental viruses (Chung et al., 2007). In the same objective, transgenic grapevines expressing the CP gene of GFLV were

exposed to nematode-mediated GFLV transmission in order to test the emergence of viable GFLV variants and recombinants. Using non-parametric estimators, Moury et al (2006) concluded that a higher diversity of GFLV haplotypes occurred in the non-transgenic rootstocks than in the CP-expressed rootstocks. These authors hypothesized that it could be due to the impact of resistance of some transgenic grapevine toward some GFLV variants. Selection of virulent variants by these transgenic rootstocks could have reduced the richness of the virus population. Conversely, no viable recombinants were obtained and this experiment did not show any modification in the genetic diversity of indigenous GFLV populations (Vigne et al., 2004a, b).

V. Conclusions and perspectives

With the dawning of the genetic engineering, a multitude of strategies have been assessed for their potential to induce resistance against pathogens in plants. These studies have also contributed to a better knowledge of the plant-pathogen relationships, hence providing valuable information for the design of new and more efficient constructs towards resistance against pathogens in transgenic plants.

Recently, microRNAs (miRNAs), which are generated from processing of longer pre-miRNAs precursors into products 20-24 nt in length (Bartel, 2004), have been identified as important regulators of gene expression in both plant and animals, in a sequence-specific manner. It has also been shown that the alteration of several nucleotides within a miRNA 21 nt sequence does not affect its biogenesis (Vaucheret et al., 2004). The possibility to modify plant miRNA sequences to target specific sequences, originally not under miRNA control, has been investigated towards protecting plants against viruses (Niu et al., 2006, Qu et al., 2007).

Once mature, these artificial miRNAs (amiRNAs) target the genomic RNAs of the plant viruses against which they were designed, and the plants transformed with the recombinant miRNA precursor became specifically immune to infection with these viruses.

Moreover, a multi-resistance strategy (combining hairpin or miRNA constructs and putative recessive resistance, e.g. eIF4 strategy, and tolerance to vectors, for example) should contribute to enhance the efficiency and durability of the resistance against the targeted diseases. This kind of associated strategies has already been described for GFLV and *X. index* (Bouquet et al., 2003, 2004). The viral transgene has been shown to be inherited in progeny issued from several crosses with transgenic *Vitis rupestris* du Lot and the rootstock 110R (Bouquet et al., 2003, 2004).

Furthermore, the availability of the whole sequence of grapevine (Jaillon et al., 2007; Velasco et al., 2007) will help to unravel the interactions between the genomic location of insertion of new transgene constructs, their regulation, the number of integrated copies and the status of resistance of the transgenic lines.

To conclude, the acceptance of transgenic strategies for resistance of grapevine towards pathogens remains a challenge. The establishment of field trials to assess the robustness and durability over years, as well as the environmental impact of pathogen-derived resistance, remains difficult for such a pluriannual crop. To assess five transgenic rootstock lines expressing the full-length translatable GFLV CP gene, in an open-field trial at INRA Colmar, an “interactive technological assessment” (Joly et al., 2004) has been proposed to pave the way and co-construct this unique experiment through a local steering commitee. Such open and pro-active dialogues between the scientific community, professionals, politicians, consumer association and the public should be greatly encouraged to shed all the light on the

benefits, limitation and practical usefullness of this biotechnology applied to grapevine resistance towards its main diseases.

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Table 1: list of nepoviruses infecting grapevines (from Digiaro et al. 2007; Le Gall et al., 2005)

***Nepovirus* genus**	Acronym	Known prevalence on grapevine
Subgroup A		
Arabis mosaic virus	ArMV	Europe
Grapevine deformation virus	GDefV	Turkey
Grapevine fanleaf virus	GFLV	worldwide
Raspberry ringspot virus-grapevine	RpRSV-Gra	Europe
Tobacco ringspot virus	TRSV	USA
Subgroup B		
Artichoke Italian latent virus	AILV	Europe
Grapevine Anatolian ringspot virus	GARSV	Turkey
Grapevine chrome mosaic virus	GCMV	Europe
Tomato black ring virus	TBRV	Europe
Subgroup C		
Blueberry leaf mottle virus	BBLMV	USA
Cherry leaf roll virus	CLRV	Europe
Grapevine Bulgarian latent virus	GBLV	Europe
Grapevine Tunisian ringspot virus	GTRSV	North Africa
Peach rosette mosaic virus	PRMV	USA
Tomato ringspot virus	ToRSV	USA
***Sadwavirus* genus**		
Strawberry latent ringspot virus	SLRSV	Europe

Table 2: list and taxonomic status of Grapevine leafroll-associated viruses (Gugerli, 2003; Martelli and Boudon-Padieu, 2006; Saldarelli et al., 2006)

***Ampelovirus* genus**	Acronym	Known prevalence on grapevine
Species		
Grapevine leafroll-associated virus 1	GLRaV-1	worldwide
Grapevine leafroll-associated virus 3	GLRaV-3	worldwide
Grapevine leafroll-associated virus 5	GLRaV-5	North Africa, Europe
Tentative species		
Grapevine leafroll-associated virus 4	GLRaV-4	Asia
Grapevine leafroll-associated virus 6	GLRaV-6	Europe, South America
Grapevine leafroll-associated virus 8	GLRaV-8	North America
Grapevine leafroll-associated virus 9	GLRaV-9	North America, Australia
***Closterovirus* genus**		
Grapevine leafroll-associated virus 2	GLRaV-2	worldwide
***Crinivirus* genus**		
Tentative species		
Grapevine leafroll-associated virus 7	GLRaV-7	worldwide

Table 3: List and taxonomic status of grapevine flexiviruses (Chabbouh et al., 1993; Monette and Godkin, 1993; Martelli and Boudon-Padieu, 2006; Martelli et al., 2007)

***Foveavirus* genus**	Acronym	Known prevalence on grapevine
Grapevine rupestris stem pitting-associated virus	GRSPaV	worldwide
***Vitivirus* genus**		
Grapevine virus A	GVA	worldwide
Grapevine virus B	GVB	worldwide
Grapevine virus D	GVD	worldwide
Tentative species in *Vitivirus*		
Grapevine virus C	GVC	Canada, USA
***Trichovirus* genus**		
Grapevine berry inner necrosis virus	GINV	Japan
***Potexvirus* genus**		
Potato virus X	PVX	Tunisia

Table 4: Diseases caused by grapevine phytoplasmas and their insect vector (OEPP/EPPO *Bull.*, 2007; Maixner, 2006; Bertaccini, personal communication).

Grapevine yellowsDisease	**Phytoplasma agents of disease**	**Taxonomic group and subgroup**	**Insect vector to grapevine**	**Geographical distribution**
Flavescence dorée (FD)	*Ca*. P. vitis[1]	16SrV-C, 16SrV-D	*Scaphoideus titanus* Ball	France, Italy, Spain, Portugal, Serbia, Slovenia, Switzerland
Bois noir (BN), Vergilbungskrankheit (VK), Legno nero (LN)	*Ca.* P. solani[1]	16SrXII-A	*Hyalesthes obsoletus* Signoret	Europe, Israel, Chile
Palatinate Grapevine Yellows	*Ca.* P. ulmi	16SrV-related	*Oncopsis alni* Schrank	Germany
Virginian grapevine yellows (VGY)	*Ca.* P. pruni	16SrIII-I	nd[2]	USA
Australian grapevine yellows	*Ca.* P. australiensis	16SrXII-B	nd	Australia
Australian grapevine yellows	*Ca.* P. australasia	16SrII-related	nd	Australia
Buckland valley grapevine yellows (BVGY)	*Ca.* P. asteris	16SrI-related	nd	Australia

[1] Both *Ca* P. solani and *Ca.* P. vitis are incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code. (Lapage *et al*., 1992)

[2] not determined

Table 5: Survey of transgenic grapevines transformed with viral CP (coat protein) genes. GCMV (*Grapevine chrome mosaic virus*).

Virus	**Host**	**Construct**	**References**
GFLV	***Vitis vinifera* Chardonnay**	cp in sense orientation	Mauro et al. 1995
	Vitis vinifera Russalka	cp in sense or antisense orientation, non-translatable cp, 5´TR cp and 3´TR cp	Gölles et al. 2000, Maghuly et al. 2006
	Vitis vinifera Nebbiolo, Lumassina Blaufränkisch	cp in sense or antisense orientation	Gribaudo et al. 2005, Gambino et al. 2005
	Rootstock Richter 110 ***(V. berlandieri x V. rupestris)***	cp in sense orientation	Krastanova et al. 1995, Laimer et al. unpubl.
	Rootstock 41 B *(V. berlandieri x V. riparia)* **Rootstock SO4** *(V. vinifera x V. berlandieri)*	cp in sense orientation	Mauro et al. 1995
	Rootstock RPG1	cp in sense or antisense orientation	Laimer et al. unpubl.
ArMV	*Vitis vinifera* Russalka	cp in sense orientation	Gölles et al. 2000
GVA	*Vitis vinifera* Russalka	cp in sense orientation	Gölles et al. 2000
GVB	*Vitis vinifera* Russalka	cp in sense orientation	Gölles et al. 2000
GCMV	**Rootstock Richter 110** *(V. berlandieri x V. rupestris)*	cp in sense orientation	Le Gall et al. 1994