Detection of VLPs by ISEM in transgenic grapevines expressing different GFLV CP-constructs

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1. ABSTRACT

Grapevine fanleaf virus (GFLV) is one of the most destructive and wide-spread viral diseases affecting grapevine (Bovey *et al.* 1980). Since virus disease control with conventional methods is very difficult and laborious, major efforts are made towards resistance breeding. Coat-protein-mediated resistance (CPmR) has demonstrated to confer a high level of resistance in herbaceous model plants (Beachy *et al.* 1990; Pacot-Hiriat *et al.* 1999) and is a promising strategy to obtain virus-resistance in perennial plants like grapevine using a pathogen derived gene. In order to produce resistant grapevines not only an efficient protection, but also environmental safety aspects have to be considered (Gölles *et al*. 2000). Possible interactions between products of the viral transgene, either RNA or protein, and an infecting virus, e.g. synergism, heteroencapsidation and recombination are considered potential risks (Tepfer 2002) and have to be prevented in any case. To develop safe transgene-constructs mutated forms of the CP gene are used, which might suppress particle assembly, heterologous encapsidation and complementation (Balázs and Tepfer 1997, Varrelmann and Maiss 2000), but still confer resistance.

These safety requirements were met by transforming grapevines with modified GFLV-CP sequences that are expected to produce smaller protein subunits unable to self-assemble to empty viral capsids. RT-PCR of the transgenic grapevines showed that CP mRNA is expressed at variable levels, but ELISA performed on leaf tissue did not show any accumulations of the GFLV CP in the analysed transgenic lines (Maghuly *et al.* 2006).

The main purpose of this work is to answer the question whether the truncated coat proteins maintain the capacity of self-assembly or not, i.e. if empty capsids (VLPs) occur in transgenic plants. For this approach ISEM was chosen as the method of analysis, because of its direct and rapid results. It has to be mentioned that the expression rate of the transgene in all plants is very low, resulting in only very few detectable VLPs. No correlation between the number of inserted transgenes and the formation of VLPs could be drawn. Five of the ten analysed transgenic plant lines (plant line 1, 2, 4, 6 and 17) showed no formation of virus-like-particles, while plant lines 5 and 17 showed only few VLPs. The two transgenic control plant lines expressing the full length CP sequence, showed few VLPs, however, much less than the virus-infected control plants.

Furthermore it could be clearly demonstrated that ISEM is a suitable method for VLP detection in CP-transgenic grapevines and that it could be recommended as a continuous standard monitoring technique for field experiments.

1. Zusammenfassung

Der *Grapevine fanleaf virus* ist einer der bedrohlichsten und weit verbreitesten Viren der Weinpflanze (Bovey *et al.* 1980). Da jedoch die konventionellen Methoden zur Kontrolle von Virus – Erkrankungen sehr schwierig und aufwendig sind, richtet sich das Hauptaugenmerk der Forschung in die Richtung der Resistenzzüchtung. Die Hüllprotein-vermittelte-Resistenz, mit der in krautigen Modelpflanzen bereits hohe Resistenzniveaus erzeugt werden konnten (Beachy *et al.* 1990; Pacot-Hiriat *et al.* 1999), ist eine der vielversprechendsten Strategien zur Resistenzzüchtung in mehrjährigen Pflanzen wie der Weinpflanze unter Verwendung eines vom Pathogen stammenden Genes. Um resistente Weinpflanzen erfolgreich zu züchten, müssen neben der Effizienz der Schutzfunktion vor allem auch die Sicherheitsaspekte für die Umwelt berücksichtigt werden (Gölles *et al*. 2000). Mögliche Interaktionen zwischen dem Produkt des viralen Trangens, entweder RNA oder Protein, und einem infektiösem Virus, z.B. Synergismus, Heteroencapsidierung oder Rekombination werden als potentielle Risiken eingestuft (Tepfer 2002) und müssen in jedem Fall verhindert werden. Um ein sicheres Transgen-Konstrukt zu erhalten werden mutierte Hüllproteingene eingesetzt, die Resistenz vermitteln, aber eine Partikelbildung, Heteroencapsidierung und Komplementation (Balázs and Tepfer 1997, Varrelmann and Maiss 2000) verhindern.

Die Sicherheitsanforderungen werden durch die Verwendung von modifizierten Hüllproteinsequenzen, deren Translationsprodukte sich nicht zu leeren Virushüllen zusammenlagern können, erfüllt. Die RT-PCR zeigte, dass in den transgenen Weinpflanzen die Hüllprotein mRNA in unterschiedlicher Stärke exprimiert wird, aber der mit Blattmaterial durchgeführte ELISA zeigte keinerlei Akkumulation des GFLV Hüllproteins (Maghuly *et al.* 2006) in den untersuchten transgenen Weinpflanzen.

Im Rahmen dieser Diplomarbeit soll gezeigt werden ob die modifizierten Hüllproteinsequenzen die Fähigkeit zum Self-assembly behalten oder nicht, d.h. ob leere Virushüllen (VLPs) in den transgenen Pflanzen gebildet werden. Auf Grund der direkten, eindeutigen und schnellen Ergebnisse wurde die Immuno-sorbent-Elektronen-Mikroskopie (ISEM) als Analysenmethode gewählt. Es muss betont werden, dass aufgrund der sehr niedrigen Expressionsrate des Transgenes, nur sehr wenige VLPs detektierbar waren. Es konnte kein Zusammenhang zwischen der

Kopienzahl des Trangens und der Bildung von VLPs festgestellt werden. Fünf der Zehn analysierten transgenen Pflanzenlinien (Linie 1, 2, 4, 6 und 17) zeigten keine VLP Bildung, während in den Linien 5 und 7 VLPs detektierbar waren. Die transgenen Pflanzen der Kontrolllinie exprimierten die komplette Sequenz des Hüllproteingens und zeigten VLPs, wenn auch deutlich weniger als die Virus-infizierten Pflanzen.

Es konnte bewiesen werden, dass ISEM eine geeignete Methode zur Detektion von VLPs in Hüllprotein-transgenen Weinpflanzen ist. Weiters ist es empfehlenswert sie als kontinuierliche Standard-monitoring Methode bei Freilandversuchen zu verwenden.

Contents

2. INTRODUCTION

Among the cultivated plant species grapevine surely is one of the most valuable ones due to the broad variety of products obtained: fresh fruit, raisins, juice and vine. Table 1 shows the systematic characterisation of this woody plant which historically has been cultivated in Europe, Middle East, Caucasus and North Africa. The preferred climate of the grapevine is the warm and mild mediterranean-like climate but it is a very flexible plant and tolerates also cooler and more humid conditions, therefore nowadays vineyards are found also in North America (California), South America (Argentina, Chile), Australia, South Africa and China. The main cultivation areas are found between the 30° and the 50° latitude degree north and south and an altitude from sea-level to 1000 meters above sea-level where the temperature never falls beyond -15°C. The kind of soil can vary from clayey to sandy, because the grapevine is a very adaptable plant. The average life cycle of a grapevine lasts 40 years and in the first three years the plants are not productive, therefore the best period for vine production is between the 5th and the 25th year of cultivation. According to the FAO about 7204300 ha of the worlds surface is dedicated to grapes which produce 66148640 tons of grapevines (FAO 2005). Approximately 71% of world grape production is used for wine (300 million hl/year), 27% as fresh fruit, and 2% as dried fruit. Table 2 lists the top wine-producers of the world and shows the corresponding areas dedicated to viticulture and the annual production of grapes.

Kingdom:	*Plantae*
Division:	*Magnoliophyta*
Class:	*Magnoliopsida*
Order:	*Rhamnales*
Family:	*Vitaceae*
Genus:	*Vitis*
Species:	*vinifera*

Table 1 Systematic characterization of the grapevine.

country	Area harvested [1000 ha]	Grape production [1000 tons]
Spain	1170,63	6066,8
France	853,91	6793,25
Italy	754,99	8553,58
Turkey	530,00	3850,00
United States	378,32	7099,18
Iran	324,55	2963,76
Argentina	211,84	2829,71
Portugal	209,77	785,35
Romania	170,98	505,85
Australia	153,77	2026,5

Table 2 Top Ten wine producers in the world ranked according to the harvested area and vine production (FAO 2005).

Among the numerous species of grapevines cultivated, *Vitis vinifera* is the mostly used for wine production. Unfortunately this species is also very susceptible to pathogens like bacteria, fungi, insects, nematodes and viruses. The diseases provoked by the infection of one, or a combination of those pathogens cause not only biological damage like stunted growth or delay in the life cycle, but also economic losses due to reduced fruit productivity or reduced quality of the grapes. These economic losses are the principal driving force for the research of protection methods. There are several conventional and effective protection methods against the principal bacterial and fungal diseases such as synthetic chemicals, special cultivation methods and the use of resistant rootstocks. For example: The covering of the vineyard to force an anticipated maturation allows the full recovery from the Phomopsis cane and leaf spot (Excoriose) caused by the fungi *Phomopsis viticola.* Against fungal diseases like Downy mildew (Peronospora) caused by *Plasmopara viticola*, bunch rot caused by *Botrytis cinerea* and Powdery mildew caused by *Unicula necator* some very effective fungicides are available on the market. Although the effectiveness and the relative low costs of fungicides, the public discussion about pesticides and human and environmental health (Spadoro and Gullino, 2005) and the relative ease with which fungicide-resistant strains emerge within vineyards drives the research efforts towards the establishment of other protection methods like resistance breeding. Curative methods like the application of chemical substances show few or no success against viruses and therefore the only conventional protection method against virus-caused diseases is prevention. Since prevention is very elaborate and time-consuming also in this field the research forces are concentrated on the development of new promising breeding techniques for pathogen-resistance against the major virus diseases of the grapevine like infectious degeneration caused by *Nepovirus*, leafroll caused by *Closterovirus* and rugose wood disease caused by *Trichovirus*.

2.1 GRAPEVINE FANLEAF VIRUS

Grapevine is susceptible to 58 viruses and 5 viroids of which more than the half is classified in genera assigned to eight different virus families and the rest is classified in genera not assigned to families (Martelli and Boudon-Padieu 2006). Besides the virus assigned to the genus *Nepovirus, Closterovirus* and *Trichovirus* which cause infectious degeneration, leafroll and rugose wood there are also a number of viruses that cause latent diseases and therefore are of no economic relevance (Prota 1996). No exact data of the economic losses due to virus diseases are available, but it is commonly accepted that they are only second behind losses due to fungi-diseases (Laimer 2006). Among the viruses that attack the grapevine the *Grapevine fanleaf virus* (for systematic classification see table 3) causing, together with *Arabis mosaic virus* (ArMV), the infectious degeneration (Bovey *et al.* 1980) is one of the most destructive ones, causing either a progressive decline of the plant vigour over several years combined with a reduction in productivity or the rapid death of young plants (Laimer 2006).

Family:	Comoviridae
Genera:	Nepovirus
Specie:	*Grapevine fanleaf virus*
Acronym:	GFLV
ICTV decimal code:	18.0.3.3.016

Table 3 Systematic classification of *Grapevine fanleaf virus* (Brunt *et al.* 1996).

The infectious degeneration, caused by the GFLV and other European Nepovirus, is also known under the name “fanleaf” because of the peculiar malformation of infected leaves, which exhibit widely open petiolar sinuses and abnormally gathered primary veins, which give the leaf the appearance of an open fan. Other synonyms are: court-noué, panachure, dégénérescence infectieuse (France), roncet, arricciamento, mosaicogiallo, degenerazione infettiva (Italy), urticado (Portugal) and Reisigkrankheit or Gelbmosaik (Germany). The disease was first described by Cazalis-Allut in 1865, but only in 1902 Baccarini suggested that “fanleaf” may be due to a virus, which

finally was confirmed by Petri in 1929. This disease, known as the most damaging one for grapevine, is caused by two major groups of biological variants i.e. distorting strains, associated with malformation of leaves and canes and chromogenic strains, associated with chrome yellow discolorations of the foliage (Martelli and Boudon-Padieu 2006).

2.1.1 VIRUS CHARACTERIZATION

Until the early 1960s *Grapevine fanleaf virus* was only partially characterized, until Quacquarelli *et al.* (1976) investigated this RNA virus with isometric particles in more detail. After the selection of a local grapevine showing typical fanleaf symptoms, the virus was propagated on *Chenopodium quinoa*, virus purification was carried out and the obtained virus purification was used for a series of laboratory tests. GFLV was fully characterized in respect to its biological, serological and morphological properties and its cryptogram completed to its present form: R/1:2.4/42+1.4/30:S/S:S/Ne.

PROPERTIES OF VIRAL PARTICLES

The genome consists of two linear single-stranded RNAs with a total genome size of 11116 kb. The larger RNA1 is 7342 nt long and has an estimated molecular weight of 2,4 x 10^6 and the smaller RNA2 is 3774 nt long with an estimated molecular weight of 1.4 x 10^6 (Quacquarelli *et al.* 1976). The strategy of expression is based on translation of both monocistronic RNAs as polyproteins that are cleaved by an RNA-1 encoded viral proteinase. The primary structure of the polyprotein (Mr of 253000) encoded by RNA 1 includes a putative RNA-dependent RNA polymerase 1E, a cystein protease 1D, the 1C protein VPg, a 1B protein containing the signatures of a nucleotide binding domain and a protease cofactor and a N-terminal 1A protein. The polyprotein (Mr 131000) encoded by RNA 2 has three final *in vitro* maturation products: (1) the 2B protein, which is the putative movement protein that accumulates in the cytosol of infected cells and whose nine C-terminal residues are critical for systemic virus spread; (2) the N-terminal protein 2A implicated in the replication of RNA-2 and (3) the 2C coat protein (CP) (Martelli *et al.* 2001). 60 subunits of this single coat protein (Mr 56019, 504 amino acids) form the polyhedral virus particles

with a diameter of 28nm. Three types of particles with different density properties, but serologically indistinguishable, have been found in purifications and named after their position in the density gradient of sucrose. The T (Top) particles have a buoyant density of 1.31 g cm^{-3} in CsCl and a sedimentation coefficient of 50 S and represent the empty shells. The M (Middle) particles with a density of 1.41 g cm^{-3} in CsCl and a sedimentation coefficient of 86 S contain RNA2 and the B (Bottom) particles with a density of 1.49 g cm^{-3} in CsCl and a sedimentation coefficient of 120 S contain RNA1 and RNA2. Electron microscopy confirmed that T components contained mainly empty virus particles which were penetrated by the negative stain, whereas M and B components consist of apparently intact particles which were not penetrated by the stain. Most of the infectivity of GFLV preparations was associated with B components, while preparations of T particles were non-infective and preparations of M particles showed some infectivity, but this may be caused by contamination with B components. The isoelectric point is pH 4 and the A260/A280 ratio is 1.67 (B), or 1.58 (M), or 0.73 (T) respectively (Quacquarelli *et al.* 1976).

SPREADING OF THE VIRUS

GFLV is a member of the Nepovirus group which name derives from: **ne**matode transmitted **po**lyhedral shaped virus particles. The virus is transmitted in a natural way by the soil-born nematode *Xiphinema index* (Hewitt *et al.* 1985) with half-persistent transmission and the vector specific transmission is determined by the viral coat protein. *X. index* feeds on root tips by penetrating into the vascular tissue with their odontostyles causing the formation of small galls. Virus particles are associated specifically with the cuticular lining of the odontophore, where the maximum concentration of particles usually occurs: the slender esophagus and the esophageal pump. Both acquisition and delivery of the virus occur within 15 minutes of feeding time and GFLV can persist in the nematode up to eight months. Vectors do not transmit virus to their progeny.

The virus is abundant in the endosperm of seeds from infected grapevines and can occasionally be transmitted to seedlings. It also occurs in pollen of infected grapevines and herbaceous hosts and is seed-transmitted in *C. amaranticolor, C. quinoa* and soybean.

The major mechanisms for virus dissemination worldwide are grafting (infected scionwood or rootstocks) and transfer of infected vegetatively propagated material. In the laboratory GFLV can be transmitted by mechanical inoculation from infected grapevine tissues to various herbaceous hosts like *C. quinoa, C. amaranticolor* and *Gomphrena globosa* (Martelli *et al.* 2001; Martelli and Boudon-Padieu 2006).

GEOGRAPHIC DISTRIBUTION

GFLV is present worldwide in all areas where *Vitis vinifera* and American hybrid rootstocks are cultivated.

2.1.2 DISEASE

SYMPTOMS ON NATURAL HOSTS

Infectious malformations are induced by the virus strains causing distorting. Leaves are variously and severely malformed, asymmetrical, show open marginal and petiolar sinuses, asymmetrical blades, irregular veins and acute denticulations. Occasionally, chlorotic mottling may accompany foliar deformations. Shoots are also malformed, showing abnormal branching, double nodes, short internodes, fasciations and zigzag growth. Bunches are smaller and fewer in number and berries set poorly, are small sized and ripen irregularly. Foliar symptoms develop early in the spring and persist throughout the vegetative season becoming less distinct in summer (Martelli and Boudon-Padieu 2006).

Yellow mosaic is induced by the chromogenic virus strains. The foliage develops bright chrome yellow discolorations early in the spring that may affect all vegetative parts (leaves, shoots, tendrils and inflorescences). Chromatic alterations of leaves vary from a few scattered yellow spots, sometimes appearing as rings or lines to extensive mottling of the veins and/or interveinal areas to total yellowing. The foliage and shots show little, if any malformation, but bunches are small and few. With increased temperatures during summer, the yellowing fades rapidly (heat masking) and the canopy develops a normal green color (Martelli and Boudon-Padieu 2006).

Crop losses range from moderate (5-10%) to very high (up to 90% or more) according to the virulence of the virus strain and varietal susceptibility. Fruit quality is also affected by a decrease in sugar content and titratable acidity. American rootstocks suffer a decrease of pruning wood up to 50% and show lower rooting ability of cuttings. There is apparently no correlation between symptom severity in grapevines and virus titer (Martelli *et al.* 2001).

CYTOPATHOLOGY

Virus particles are found in roots, mesophyll and vascular parenchyma in herbaceous hosts or grapevines. Histological observations have pointed out the localization of virus particles in the cytoplasm and the nucleus of the infected plant cells (Savino et al. 1985) where they accumulate in form of crystalline aggregates. In tissues of experimentally infected herbaceous hosts (*C. quinoa, C. amaranticolor, N. clevelandii, P. hybrida*) aggregates of virus particles are common next to, or inside, large inclusion bodies (vesiculate-vacuolate inclusions) consisting of ribosomes, endoplasmic reticulum strands, and membranous vesicles containing fine fibrils. In these inclusion bodies the virus particles are usually arrayed in long straight parallel rows forming true crystalline or sometimes in curved superimposed rows forming paracrystalline aggregates. True membranous tubules containing rows of virus particles are connected to plasmodesmata, or are present within cell wall protrusions that develop at the level of plasmodesmata indicating a mode of short distance spreading of GFLV in its host. In *N. clevelandii* virus particles were only observed in the vacuole indicating an active mechanism of discarding from the cytoplasm which is a possibility for the plants to get rid of the virus particles in the cytoplasm (Gerola *et al.* 1969; Saric and Wrischer 1975; Savino *et al*. 1985). The virus replicates in the cytoplasmic inclusion bodies whose membranous vesicles are thought to be the site of viral polyprotein processing and RNA replication (Pfeiffer *et al*. 2000). Endocellular cordons (trabeculae), i.e. abnormal ribbon-shaped straight bodies made up primarily of cellulose, cross the lumen of vascular elements of infected grapevines (Martelli *et al.* 2001).

DIAGNOSIS

Although it requires space, time, an exact knowledge of the symptoms and a lot of experience, mechanical transmission on diagnostic species is still a frequent used diagnostic technique, because it is still regarded necessary for the certification of the freedom of virus infection. *Vitis rupestris* St. George reacts within 3-4 weeks following graft inoculation with chlorotic spots, rings and lines and localized necrosis (shock symptoms). Chronic symptoms are reduced growth, severely deformed leaves with prominent teeth or yellow discolorations and mild deformations of the leaves, depending on whether the inoculum is a distorting or a chromogenic virus strain. *Chenopodium amaranticolor* and *C. quinoa* develop chlorotic/necrotic local lesions within seven to ten days after the mechanical inoculation by infected sap. Systemically infected leaves show mottling, vein clearing, and deformation depending on the virus strain. Infected plants are stunted, but the symptoms fade as the plants age. *Gomphrena globosa* shows chlorotic local lesions within 7-8 days after inoculation, which soon turn into reddish, light green or yellow spots and twisting of systemically invaded upper leaves. *Nicotiana benthamiana* and *N. clevelandii* show occasional faint yellowish lesions followed by systemic mottling and deformation of the leaves in 10 to 15 days after virus inoculation (Martelli *et al.* 2001). *Cucumis sativus* and *Phaseolus vulgaris* cv. Bountiful show systemic chlorotic or necrotic mosaic, mottling, flecking or ring spots, but can be immune to some strains (Brunt *et al.* 1996).

ELISA using polyclonal antisera and monoclonal antibodies is a quick, cheap and very sensitive method. Molecular hybridization assays using radioactive or digoxigenin-labelled probes, RT-PCR and immunocapture RT-PCR are becoming increasingly popular. ELISA and/or RT-PCR are most successfully carried out on samples of young leafs or phloem. Other techniques to identify virus infection in plants are the immunosorbent electron microscopy (ISEM) and gold labeling techniques (Martelli and Boudon-Padieu 2006).

Trabeculae or endocellular cordons, i.e. radial bars crossing the lumen of epidermal, parenchyma, phloem and xylem cells, can be a useful indication of an infection with GFLV, but is not a specific test. These structures are readily visible by light microscopy in lignified shoots, especially in the basal internodes (Martelli and Boudon-Padieu 2006).

DISEASE CONTROL AND PROTECTION METHODS

Since against viral diseases no effective curative methods are available, the main efforts have to be concentrated on vector control, prevention of infection and resistance breeding. Conventional methods of GFLV control are based on cultural practices (rouging, fallow) to reduce the source of inoculum and on the use of agrochemicals against nematode vectors to reduce virus spread.

The virus persists mainly in *X. index,* in grapevines and in the roots of lifted grapevines that remain viable in the soil and therefore constitute an important source of virus inoculums. Infected seedlings and weeds have minor epidemiological significance, although *X. index* was able to acquire the virus from the roots of *C. amaranticolor* and subsequently transmit it to *V. rupestris.* The vertical distribution of *X. index*, which is the major, most efficient and economically important vector, in the soil follows closely that of the host root system and the populations in temperate climates are not much affected by soil temperatures (Martelli *et al.* 2001). In contaminated soils, the use of fumigants against nematode vectors gives only a temporary control of the disease and is more and more questioned for environmental reasons. The use of efficient chemical substances like dichlorpropen has currently been forbidden because of the high toxicity of the chemicals and their cancerogenic properties (Spielmann *et al.* 1997). It is highly recommended to realize new plantations on nematode free grounds. Local virus spread is difficult to control, because cultural practices such as prolonged fallow, crop rotation, tillage and weed control are equally of little effect.

Another attempt to limit the virus spread is to prevent the infection of the plants by cultivating virus-resistant crop plants. The development of transgenic grapevines resistant to GFLV by integration of a viral gene is a promisingly strategy and has been used till now mainly to engineer the virus coat protein gene into rootstocks and *V. vinifera* lines (Krastanova *et al.* 1995; Mauro *et al*. 1995; Spielmann *et al.* 1997, 2000a; Xue et al. 1997; Gölles *et al.* 1998). Under experimental conditions, delayed infection or lower virus titre have been observed in some of the transgenic grapevine lines (Barbier *et al*. 1997; Courtois *et al*. 1997; Spielmann *et al.* 1997) but these results still need to be evaluated in the field. While constructs containing the full length CP sequence, either in sense - (Moser 1997) or antisense – orientation or

untranslatable sense – RNA (Schlangen 2000) demonstrated to confer resistance to GFLV infection in herbaceous hosts, the ability to confer resistance in transgenic grapevines has still to be examined. On the other hand, field trials demonstrated the resistance of transgenic grapevines expressing the full length CP sequence of GFLV against virus infection (Fuchs et al. 2000; Vigne *et al.* 2004a, 2004b).

Long distance spread can be controlled by the production and distribution of healthy propagation material and the use of certificated virus-free scionwood and rootstock materials for new implantations (Savino 1996). With the help of sanitary selection and the application of recovery techniques like thermotherapy (38-40 °C for 4 weeks), micrografting, *in vitro* meristem tip culture or somatic embryogenesis virus free material can be obtained (Prota 1996). In order to prevent the further virus spread and to ensure the quality of the propagation material to the winegrower the “European and Mediterranean Plant Protection Organization” (EPPO) has approved a certification scheme for grapevines. It describes the steps to be followed for the production of vegetatively propagated healthy planting material (varieties and rootstocks) and includes also guidance on testing procedures for virus and virus-like diseases which should be tested for absence (GFLV, GVA, GVB, GLRaV 1-9 etc.) (EPPO 2003).

2.2 PROTECTION AGAINST VIRAL DISEASES

Before considering protection against viral diseases it is necessary to remember that (1) up to now no chemical substance is available that administered to a virus infected plant allows the recovery; (2) vegetative propagated material obtained from a virus infected plant results infected in almost every case; (3) infectious agents are spread in a natural way by vectors like nematodes and insects from one plant to another. Since no therapeutic substances are effective to fight against virus–caused-diseases, because the virus-host interactions are so tightly related that a substance inhibiting the virus will nearly always also damage the host cell, the only way to fight against virus diseases is: prevention. There are several strategies of preventive–disease-control and phytosanitory regulations to inhibit the spreading of virus diseases including a) the eradication of infected plants and the realization of new implantations only with certified virus-free plants; b) the repeated and extensive use of chemicals to control the virus vectors and c) the introduction of natural resistances against the virus or its vector into the plant (Savino 1996).

2.2.1 CONVENTIONAL METHODS: PREVENTION, RECOVERY AND CERTIFICATION

At the moment the only real strategy of efficient struggle against virus diseases is prevention of infection based on vector control, use of resistant rootstocks and the use of certified material which is obtained across sanitary selection and/or recovery techniques. In order to facilitate the production of certified material and to prevent the further virus spread standard regulations on national and international level have been enacted. Their aim is the regulation of the production and commercialising of multiplication material and insurance for the winegrowers by certificating the sanitary and variety state of the plants. The investigation of the sanitary state is carried out with rapid and sensible diagnostic techniques like ELISA and RT-PCR that provide reliable results and are also able to identify the etiologic role of the virus towards the plant.

PREVENTION

Interventions with preventive character are e.g. agronomical measures easy to apply and addressed to the elimination of infection possibility for already existing plant cultures, fitoiatric measures against vectors and the production of virus-free propagation material which is used for the certification programme.

Agronomical measures exists that if used with reason and in combination are efficient, economic and do not disturb the environmental balance. However exact knowledge of epidemiologic cycles, virus impact on the plant's physiology and plant-vector-virus interactions are necessary to obtain satisfying results. While in case of horticultural cultures rotations, anticipated sowing and/or transplant or the interruption of the cultivation for a certain period of time are quite efficient techniques, for multi-annual fruit trees and grapevine the possibilities are limited. Therefore the elimination of culture residues and of weeds, which represent a reservoir of infection and a nutrition resource for vectors, is highly recommended. In case of soil-born vectors like the nematode *X. index* for *Grapevine Fanleaf Virus*, the only accurate way of defence is to remove all parts of the grapevine and to elaborate the soil for at least two years till soil analysis confirm the absence of nematodes or in alternative carry out a rotation of cultivation with cereal crops.

RECOVERY

The recovery methods for plant material infected with viruses or virus-like-agents available nowadays are numerous, show good efficacy and are often combined among them.

The thermotherapy is the exposure of the infected plant material to dry or humid heat for a certain period of time. The major effects are a blockage or slowdown of the virus replication and off the systemic virus spread due to damages of the coat proteins and the nucleic acid and blockage of ribosomes due to a competition between genomic RNA and virus RNA. As a consequence of the reduction of virus replication and migration, caused by the higher temperature, newly emerged tissues of the infected plant can be virus-free. This method allows the recovery of vegetative apices and buds from infected plants, which, if cultivated under higher temperature conditions and micropropagated, can develop into new virus-free individuals. The *in vitro* culture of apical meristems forecasts the sterile cultivation of meristematic explants taken

from apical or lateral buds. The absence of virus in the meristematic regions seems to be depending on various factors like the lack of vascular connections at the tip of the meristem, the increased replication speed and the increased metabolism of these cells which do not allow any additional replication e.g. virus replication. The success of this recovery technique depends on the explants dimensions, the virus type and the genotype of the plant. The smaller the explant is, the more likely is successful recovery but the lower is the regeneration of the explants. The technique of micrografting is an alternative solution for plant species that do not tolerate higher temperatures and/or for which *in vitro* regeneration of apical buds is very difficult. Explants of meristematic apices of the infected plant are grafted in vitro onto a healthy and compatible rootstock. Due to the poor knowledge of viral replication mechanisms and to the difficulties of finding an antiviral substance that is economic and not toxic for the plant cells, the chemotherapy for recovery is one of the less used ones.

CERTIFICATION

By certified propagation material is meant material which derives directly from progenitor plants that have been obtained via clonal and sanitary selection, eventual recovery, and analyzed one by one for the virus absence. Since the certification is a technical-legislative procedure following precise protocols defined by the responsible disciplinarians it is able to guarantee the genetical quality and the sanitary state of vegetable propagation material. However the sanity of the certified material cannot be understood in an absolute way, but can vary from species to species and from country to country as it depends on the predefined standards. It appears more reasonable and technical easier to pursue only the exemption of viruses that cause economical important damage. At the European level, the current legislation intends that a certified grapevine has to be free of the causal agents of infectious degeneration (i.e. GFLV and other European *Nepoviruses*) and leafroll (*Closteroviruses*) (Savino 1996). The EPPO has approved and published certification schemes for all important cultivated plants, which are continuously improved and updated (EPPO 2003).

2.2.2 RESISTANCE BREEDING

Resistant plants might be the most convenient protection methods for long-living horticultural crops such as fruit trees and grapevine. However, conventional breeding methods are time consuming and often also difficult to realize due to genetic barriers and the complexity of fruit trees, the long breeding cycles and the difficulties of combining all other desired horticultural features with virus resistance (Laimer 2006). The improvement of molecular-biological techniques and the increased knowledge of virus genetics have lead to the development of engineered protection methods which offer a totally new way of plant protection and on the top of that are less time consuming. Two different types of introduced resistance are distinguished according to the origin of the resistance gene: host–gene-mediated virus resistance and virus-gene-mediated virus resistance. In the first case a gene that confers resistance in a wild species of the same genus or in another plant family, is genetically introduced into the crop plant.

Due to the low efficiency and high costs of conventional control methods, GFLV-resistant grapevines offer a new road for convenient virus protection. Currently the effort is concentrated in different laboratories to create GFLV-resistant rootstocks or cultivars trough traditional breeding methods or genetic transformation technology. Almost all known *Vitis vinifera* L. varieties are susceptible, but variable levels of sensitivity and tolerance to infection are widespread in European grapes. A high resistance level of the host plant resistance type was found in two accessions from Afghanistan and Iran. This resistance to *X. index* and/or GFLV is controlled by two unlinked recessive genes (Walker and Meredith 1990). Resistance to *X. index* feeding was identified in several *Vitis* species including *V. rotundifolia, V. rufotomentosa, V. munsoniana* and some American rootstock hybrids. Muscadine grapevines (*V. rotundifolia*) are highly resistant to virus transmitted by nematode feeding but not by graft-inoculation with infected scions (Martelli and Boudon-Padieu 2006). Until now, traditional breeding methods have not yet allowed the release of new cultivars truly resistant to GFLV and/or its vector (Laimer 2007). Also, this approach has not been used much up to now, because the identification and characterisation of host-resistance-genes is very difficult (Laimer 2006) and viral genes are more accessible.

PATHOGEN DERIVED RESISTANCE ASSESSMENT

Sanford and Johnson (1985) first suggested to introduce a pathogen-derived gene into the plant. They proposed that the resistance is obtained due to the expression of the viral gene product either at an inappropriate time, an inappropriate amount or an inappropriate form during the infection cycle, which inhibits the pathogens ability to maintain an infection. Plants transformed with pathogen–derived–resistance-mediating genes show a broad range of resistance phenotypes that vary from a delay in normal symptom development across partial inhibition of viral replication to complete immunity to resist virus or viral RNA inoculation (Wilson 1993). The mainly used pathogen-derived genes are those for coat proteins (CP), replicases, defective interfering RNAs and DNAs, movement proteins and non-translatable RNAs. The strategies of pathogen-derived-resistance (PDR) based on the accumulation of viral nucleic acid sequences normally lead to a very high level of resistance against one specific virus strain, while the resistance that is conferred by the accumulation of the protein leads to resistance against a wider range of virus strains and viruses (Beachy 1997). The targeted post–transcriptional breakdown of RNA sequences, also called RNA mediated suppression, is one of the most studied possibilities of nucleic acid mediated resistance and normally leads to a very high level of resistance against viruses which contain genome sequences homologous to the transgene's sequence (Baulcombe 1996). An effective and very specific resistance of transgenic tobacco plants to the infection with *Bean yellow mosaic potyvirus* (BYMV) was obtained by the expression of antisense RNA containing the carboxy–terminal portion of the BYMV coat protein, the complete 3'-noncoding sequence and a short poly(A) tract (Hammond and Kamo 1995). RNA-mediated resistance was also reported for *Cucumber mosaic virus* (Chen *et al.* 2004) and for *Plum pox virus* (Hily *et al.* 2005) using inverted repeat (IR) constructs. A new and promising approach is the creation of a multiple virus resistance due to the use of a single IR construct, containing several fragments of different viral sequences (Bucher *et al.* 2006). The mechanism of replicase-mediated resistance (Rep-mR) is not yet elucidated, although it is very probably that the protein produced by the expression of the transgene interferes with the replicase produced by the virus. The effectiveness of replicase-mediated resistance to *Cucumber mosaic virus* was increased by transgene translatability (Wintermantel and Zaitlin 2000). The replicase-mediated resistance, generally limited to the virus strains from which the transgene sequence was obtained, may occur by

binding of the transgene protein to host factors or virus proteins that regulate the virus replication. The expression of full–length *Cucumber mosaic virus* (CMV) RNA2 prevented both the accumulation and systemic infection of CMV in transgenic tobacco plants demonstrating the two independent replicase–mediated resistance mechanisms: resistance against viral replication and resistance against movement (Hellwald and Palukaitis 1995). Transgenic tobacco plants expressing a defective mutant of the *Tobacco mosaic virus* (TMV) movement protein showed resistance not only to several *Tobamoviruses*, but also to representatives of the *Potexvirus*, the *Cucumovirus* and the *Tobravirus* groups. These results indicate that there are similar functions among the movement proteins of different virus groups and that movement protein–mediated resistance is effective against a broad range of viruses with the use of a single transgene (Cooper *et al.* 1995).

COAT PROTEIN MEDIATED RESISTANCE

Among the various nucleic acid sequences of plant viruses that have been introduced into plants in order to produce resistance, the ones encoding coat proteins have shown to be particularly useful. A phenomenon like cross protection, which is the reduced susceptibility of a plant infected with a mild strain of a given virus to infection with a virulent strain of the same virus, can be mimicked in genetically engineered plants accumulating viral coat protein. Coat-protein-mediated–resistance (CPmR) is defined as the resistance which is acquired due to the expression of a virus coat protein gene in transgenic plants. This stably inherited resistance to the virus from which the coat protein gene was derived seems to be mediated by the accumulation of virus coat protein and inhibits viral infection or disease development (Beachy *et al.* 1990). The different levels of resistance conferred by this strategy, varying from immunity to delay and symptom attenuation, indicate that both protein- and RNA-mediated-protection mechanisms could be involved (Prins *et al.* 2007). Considering the diversity of roles of the CP in different plant/virus systems, it is not surprising that the mechanisms of resistance in CP-transgenic plants are also remarkably diverse. Since the mechanisms of the CP-mediated-resistance are not yet fully discovered different theories are supported: (1) the transgenic CP mRNA may anneal with the (-) strand of the challenge virus thus preventing replication of the genome; (2) the sequence at the 3’ end that forms a tRNA like structure may bind the

replicase and inhibit the replication of the challenge virus; (3) the coat protein may interfere with an infection stage (Powell *et al.* 1990). The transgene CP might prevent virus disassembly (Sherwood and Fulton 1982), recoat the RNA as it is stripped (De Zoeten and Fulton 1975) or interfere with a putative receptor site on the host (Lomonossoff 1995).

The first practical evidence of CPmR was provided by Powell *et al.* in 1986 which reported the resistance of transgenic tobacco plants to *Tobacco mosaic virus* (TMV). They demonstrated that plants regenerated from tobacco cells previously transformed with a gene encoding the coat protein of the TMV failed to develop symptoms or showed at least a delay in symptom development. Nelson *et al.* (1987) compared the symptom development and the virus accumulation in transgenic CP(+) tobacco plants with those in corresponding CP(-) plants after the infection with TMV. They observed 95-98% fewer necrotic local lesions on the CP(+) plants than on the CP(-) plants after the inoculation with the virus. They also reported that the inoculated leaves of the CP(+) plants contained 70% less TMV than those of the CP(-) plants and that nearly no virus was found in the new leaves that emerged after the inoculation. Other studies showed comparable results for *Tobacco etch virus* (Lindbo *et al*. 1993) and *Tomato black ring nepovirus* (Pacot-Hiriat *et al.* 1999) in transgenic tobacco plants. Powell *et al.* (1990) reported that the protection against TMV infection was conferred by the accumulation of the coat protein rather than by CP mRNA and they also refuted the theory, at least for TMV, that the viral replicase is sequestered by the transgene transcript, because deletion of the sequence for the tRNA-like structure did not affect the level of protection. Yusibov and Loesch-Fries (1995) determined that the high-affinity RNA-binding domains of *Alfalfa mosaic virus* CP are not required for CPmR thus excluding the importance of interactions between transgenic CP and virus RNA for protection. Experiments with TMV coat proteins mutated in a way that affects the subunit-subunit interactions showed that the capacity of assembly is necessary to confer resistance, because mutants not capable of subunit-subunit interactions did not confer resistance while the ones with increased subunit-subunit interactions provided a higher protection than that conferred by wild-type CP (Bendahmane *et at.* 1997). The state of aggregation of the transgene coat protein plays a role in CPmR, suggesting that CPmR may depend on certain quaternary structures rather than on the CP itself (Asurmendi *et al.* 2007). The strength of CPmR to TMV infection in transgenic *Nicotiana tabacum* has been

reported to depend on the degree of regulation of replication by aggregates of the CP (Bendahmane *et al.* 2007).

Approaches towards GFLV resistance in transgenic grapevines with CPmR have been made by several scientists either with full length CPs in sense orientation (Mauro *et al.* 1995; Krastanova *et al.* 1995; Gölles *et al*. 2000) or with mutated forms (Gölles *et al.* 2000; Gribaudo *et al.* 2003; Gambino *et al.* 2005; Maghuly *et al.* 2006). The results for full length transgenes vary from delay in symptom development (Spielmann *et al.* 1997, 2000a) to fully resistance (Moser 1997) in transgenic tobacco plants, while in transgenic in vitro grapevines no protection to GFLV infection via green grafting, micrografting, nematode transmission (Barbier *et al.* 1997; Spielmann et al. 1997, 2000a) or electroporation of virus into grapevine protoplasts (Mauro *et al.* 2000) was reported. On the other hand, a four year field evaluation of transgenic grapevines indicated that the expression of the CP gene of GFLV can exhibit a promising level of resistance against GFLV (Fuchs *et al.* 2000). 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 confirming that transgenic grapevines are likely to be of practical interest for the control of GFLV. The study further indicated that transgenic grapevines did not favour the development of GFLV recombinant isolates to a detectable level. Thus, GFLV-resistant transgenic grapevines could allow sustainable production while preserving the environment (Vigne *et al*. 2004a, 2004b).

More research is needed now to assess the sustainability and stability of the engineered protection. However, based on the successful protection of herbaceous hosts with the same transgenes as those expressed by transgenic grapevines (Bardonnet *et al*. 1994; Monier *et al*. 2000; Martinelli *et al.* 2000; Radian-Sade *et al.* 2000; Spielmann *et al.* 1997, 2000a), there is little doubt that transgenic grapevines exhibiting high levels of resistance to viruses will be obtained (Fuchs 2003).

2.3 ELECTRON MICROSCOPY

The first electron microscope (EM) prototype was built in 1931 by the German engineers Ernst Ruska and Max Knoll, patented by Siemens and capable of magnifying objects by four hundred times (Ruska 1986). Modern electron microscopes are still based upon Ruska's prototype, but can magnify objects up to two million times and are applicable also for the examination of biological materials like microorganisms, cells and viruses. For the observation of viruses a Transmission electron microscope (TEM), involving a high voltage electron beam emitted by a cathode and focused by electrostatic and electromagnetic lenses, is used. The electron beam transmitted through a specimen that is in part transparent to electrons, carries the information about the inner structure of the specimen. Since the first EM picture of a plant virus (showing TMV) was published (Kausche *et al.* 1939), the classification of plant viruses has been based mainly on the morphology of their particles visible in the EM. From there on electron microscopy has been used in the initial stages of detection or identification of a plant virus because of four advantages: (1) procedures are simple, rapid and inexpensive; (2) virus particles do not resemble anything occurring in a crude extract of a healthy plant; (3) their shapes and sizes are highly diagnostic; and (4) their presence is revealed without preconception. Another very useful application is the real-time monitoring of virus purification steps, as it allows the rapid and exact examination of the fractions determining where and in which concentration the virus is present (Milne 2006).

Immune-electron microscopy (IEM) is a technique that detects the specific binding of antibody to antigen by electron microscopy and the first observation of virus-antibody interactions in the EM was made by Anderson and Stanley in 1941. Since immune-EM techniques combines the advantages of the electron microscope (immediacy and fine spatial resolution) and serological tests like ELISA (high specificity), they can be powerful, quantitative and more rapid than other methods if the number of samples is limited. However, the routine use of EM is limited, because although the EM can image biological structures and resolve them with separations of one or two nanometers, the processing of many samples becomes slow and laborious. Therefore the immune-electron microscopy is mainly used to resolve particular problems, not for mass screening (Milne 1993).

2.3.1 Obtaining Contrast: Negative Stains (Hayat and Miller 1989)

Virus particles and antibodies are biological material composed of atoms of relatively low atomic number and do not scatter electrons very effectively. They are almost invisible in the EM and have to be stained with heavy atoms to give a good contrast. By negative staining the object is rinsed with a heavy metal compound, giving a dark background with the particles showing up in white (Savino *et al.* 1985). This method is simple, quick and acts also as sustain for the particles, making the fully dried grids storable for a long period of time and protecting them against flattering and the electron beam. Several different stains are available, but a 1-2% solution of Uranyl acetate (UA) in water is the most used and convenient stain. The stain is poisonous, if ingested and mildly radioactive, emitting alpha, beta and some gamma rays, which do not pass through the walls of a normal glass bottle. UA keeps well in a dark bottle at room temperature for several weeks, contrast is high and resolution is good and it is safe for most of the virus preparations. The only disadvantages of UA are the relatively low pH of 4.2-4.5 and the fact that it precipitates in contact with plant sap or phosphate. Phosphotungstic Acid (PTA) is used in a 1-2 % solution and its pH near 7 is adjusted with NaOH or KOH. Unlike UA this stain does not precipitate in contact with phosphate or plant sap, but damages a large variety of plant viruses among them *Cucumoviruses*, some *Geminiviruses*, some *Ilarviruses, Alfalfa mosaic virus, Rhabdoviruses*, some *Reoviruses*, *Tomato spotted wilt virus* and some *Closteroviruses* (Francki *et al.* 1984). Sodium Silicotungstate, used as a 1-2% solution of the acid in water with pH 7, forms smaller microcrystals than UA or PTA and can resolve details separated by only 1 nm. Ammonium Molybdate (AM), used in a 2% aqueous solution with a pH range from four to nine, causes only little damage to virus preparations and an can be added directly to the preparation. Its flexibility sometimes is very useful, but due to its low level of contrast it is not a good choice for routine analyses (Milne 1993).

2.3.2 IMMUNOSORBENT ELECTRON MICROSCOPY (ISEM)

ISEM is an electron-microscopy technique involving immune-trapping of the virus on a solid support, further immune-labelling of the immobilized particles and finally observation of the results in the EM. The combination of two immune-reactions leads to the best results obtainable, because the first reaction traps only the virus out of non-purified sap from infected plants and therefore increases specificity and the second confirms which virus has been caught (Milne 1977). ISEM is used to realize several aims: (1) to detect specific virus particles in a given specimen; (2) to estimate the degree of serological relationship between virus isolates; (3) to identify specific antibody-binding sites on viral particles and (4) to titrate antisera or other antibody preparations (Milne 1993). In all these applications the advantages are the good sensitivity, the brief processing times, the small reagent volumes, the (apart from the electron microscope facility itself) simple equipment and of course that the positive result is very easily and safely to interpret. False positives or negatives are really rare because you see the virus particles, the antibody attached to them and also how much antibody and where it is attached.

VIRUS PREPARATION

The sample can either be leaf tissue, not lignified roots and shoots or more specifically phloematic tissue. Due to the specific antigen- “fishing” of the immobilized antibodies the crude plant extract can be used directly after the homogenization of the sample in a suitable buffer. If necessary, the crude extract can be centrifuged for a few minutes at low speed and the clarified supernatant is used for the incubation.

ANTIBODY COATING AND ANTIBODY DECORATION

When particles adsorb to a surface, the curve of particle concentration against time is usually asymptotic, i.e. particle first adsorb rapidly till a critical point, while longer incubations beyond it yield no increase in particle concentration. In practice longer incubation periods are used to ensure that the asymptote has been reached, where small errors in incubation time do not cause significant differences in the adsorption intensity (Milne 1993). Nevertheless, the state where the original surface is saturated and more than one layer of the bound particle begins to develop due to protein-

protein interactions should be avoided, because it only increases instability (Cantarero *et al.* 1980).

Coating the grid with a specific antibody allows the use of crude plant extracts, which decreases labour for sample preparation enormously. Decorating the trapped virus with another layer of antibody allows many more applications: (1) rendering the virus particle more conspicuous by increasing its size and contrast; (2) confirming the serological identity of a virus and detecting or confirming the presence of mixtures of serologically different but morphologically similar viruses; (3) measuring of antisera titre; (4) estimating of the degree of relationship between viruses and (5) localizing of particular antigens on the viral surface (Milne 1993).

VIRUS-TRAPPING

Short incubations of about 15 minutes are usually done at room temperature and are sufficient to trap enough viruses for qualitative answers, but do not reach the maximum sensitivity. Longer incubation periods vary from three hours to overnight either at 37°C, room temperature or 4°C and increase the sensitivity. However, overlong incubations may lead to particle degradation or detachment of particles already trapped. For longer incubation periods at room temperature or 37°C, substances that inhibit bacterial growth (i.e. sodium azide) and proteolytic enzymes must be added to the preparation and the grid must be prevented from drying out. Although overnight incubation at 4°C often is a convenient option, incubation time and temperature must be optimized for each system (Milne 1993). In case of the GFLV, i.e., extremely long incubation times are needed, i.e. best results where obtained after 8 days of incubation at 4°C, when 270 times more virus was trapped than after 15 minutes (Bovey *et al.* 1980).

NEGATIVE STAINING

In order to facilitate the visualization of the virus preparation the decorating antibodies are negatively stained by rinsing the grids with 2% UA (or other stains like PTA) or alternatively by incubating the grids on a drop of the stain for 30 seconds and then dried them on air. The stain not only surrounds the specimen duo to surface tensions, but also penetrates into hydrophilic regions of the specimen to replace

water. Since most proteins are charged negatively at neutrality, there is little attraction between anionic PTA and proteins. Uranyl cations (UO_2^{2+}) used at pH 4.5 also do not interact with proteins, because at this pH most proteins are positively charged. Areas of negatively stained specimens that have more protein generally exclude stain and appear light, whereas areas with less protein allow stain penetration and appear dark (Hayat and Miller 1989).

PLANT VIRUS DETECTION WITH IMMUNOSORBENT-ELECTRON MICROSCOPY

Since ISEM is applicable for every virus against which a sufficient sensitive antiserum can be produced, it is often used as a visual confirmation of results obtained with other serological or molecular methods for known viruses. In the original ISEM paper the method was also proposed as a quantitative approach (Derrick 1973). Other applications are the distinction between related viruses and the demonstration of mixed infections or of unknown viruses in an extract. It is also a very suitable method for analyses concerning the better understanding of the localization of virus particles in infected host cells and of the alterations caused at an ultrastructural level (Gerola *et al.* 1969).

A new application is the support of risk assessment studies regarding transgenic plants by ISEM where it may dissipate concerns by demonstrating the safety of certain transgenic plant lines or point out risky areas where precautions should be taken. The occurrence of heterologous encapsidation in transgenic tobacco plants which leads to aphid transmission of a non-aphid transmissible *Zucchini yellow mosaic virus* (ZYMV) strain has been demonstrated with the help of ISEM (Lecoq *et al.* 1993). No pseudo-particles of *Tomato black ring nepovirus* were detectable by ISEM in transgenic tobacco plants expressing a truncated form of the coat protein, therefore confirming these plants as safe regarding heterologous encapsidation and suitable for further breeding studies (Pacot-Hiriat *et al*. 1999). On the other hand empty virus-like particles similar to empty virus shells have been detected by ISEM in recombinant baculovirus-infected insect cells and transgenic plant cell expressing a modified coat protein of *Arabis mosaic virus* (Bertioli *et al.* 1991) and in transgenic *N. benthamiana* expressing the CP of ArMV (Spielmann *et al.* 2000b).
In general a visual and rapid method like ISEM is extremely important and useful for the specific examination of molecular structures like either virus in infected plants or VLPs in transgenic plants expressing CP sequences.

2.4 RISK ASSESSMENT

Virus resistant transgenic plants hold the promise of enormous benefit for agriculture and the use of pathogen derived genes opens an enormous source of virus resistance genes that could be used in crops where sources of natural resistance are inadequate (Tepfer 2002). Most examples of PDR for plant viruses are RNA mediated and occur through the mechanism of posttranscriptional gene silencing (PTGS), which is also now commonly referred to as the antiviral pathways of RNA silencing (Lindbo and Dougherty 2005). Nevertheless numerous studies showed that transgenic plants with viral CP indeed provided specific resistance against the viruses with identical or similar CPs (Powell et al. 1986; Beachy *et al.* 1990; Pacot-Hiriart *et al.* 1999; Spielmann *et al.* 2000a; Vigne *et al.* 2004b). Transgenic resistance is the most effective way of controlling plant viruses for the following reasons: (1) virus resistance can be incorporated into a plant without changing its intrinsic phenotypic properties, something that is virtually impossible to achieve with conventional breeding; (2) the same resistance gene can be incorporated into different plant genera and species that are affected by a given virus and are amenable to transformation and regeneration; and (3) resistance can be incorporated into vegetatively propagated plants (Fuchs and Gonsalves 2007). Due to several characteristics of viruses and their replication already very early assessments of the possible dangers of the release of virus-gene-transgenic plants in the environment arose (de Zoeten 1991). Numerous studies in the last 20 years have addressed this topic, but generally focused more on the occurrence of potential risks than on the consequences of these occurrences. The sources of potential concerns are the engineered trait (e.g. virus resistance) and the transgene (e.g. a virus-derived gene construct) and therefore, it is critical to determine a baseline level of occurrence against which the impact of transgenic plants is compared (Fuchs and Gonsalves 2007). Identifying risks and assessing their impact on the environment is a necessary prerequisite for the safe deployment of virus-resistant transgenic plants and is a particularly relevant issue in the case of a perennial crop like grapevine. Since the grapevine remains in the field for many years the probable occurrence of unintentional phenomena such as recombination, heteroencapsidation, complementation or transgene dissemination through pollen flow increases (Fuchs 2003).

2.4.1 PRINCIPLE RISK ISSUES

Potential safety considerations relate directly to the fact that resistance to viruses in plants is achieved through expressing constitutively viral sequences, which normally do not occur in conventional plants (Fuchs and Gonsalves 2007). The areas of potential risk can be divided into two classes depending on whether the genome of either partner in the virus-host interaction is affected. The first group compromises all situations in which the phenotype of the plant-virus interaction is modified, but the genotype of neither partner is affected. This includes various forms of complementation, heteroencapsidation and synergy, which essentially are reversible. The second group covers potential risks mediated by genotypic changes of either the plant or the virus, including mutational drift in satellite RNAs, plant-to-plant gene flow by out-crossing and plant-to-virus gene flow by recombination. These effects are of greater concern because they are potentially irreversible (Tepfer 2002).

POTENTIAL RISKS DUE TO PHENOTYPIC EFFECTS

Complementation phenomena can lead to changes in virus movement, tissue specificity and host range. It has been shown that transgenic tobacco plants expressing a TMV CP sequence were able to complement a TMV CP defective strain leading to the formation of infectious virus particles and long range spread of infection (Osburn *et al.* 1990). Early arisen objections whether virus resistant plants expressing a coat protein could lead to symptom amplification, changes in tissue specificity or host range in case of infection with another virus could be calmed because such effects would be noticed early in the development of the virus resistant transgenic plant, thus leading to the modification or rejection of the resistance gene.

Heteroencapsidation is the heteroencapsidation of the genome of one virus by the coat protein of another virus, i.e. viral RNA can be encapsidated in particles composed entirely or partially of the coat protein of another virus. Various forms of heteroencapsidation can be observed when plants are infected with two closely related viruses, potentially leading to changes in vector specificity (Tepfer 2002). Since it was pointed out that transgenic CP was able to complement an infecting virus, also the possibility of heteroencapsidation in CP-transgenic plants must be considered. The properties of heteroencapsidated viruses might change, because the CP can carry determinants for pathogenicity and vector specificity. As a result of heteroencapsidation and subsequent vector-mediated transmission a virus could

infect an otherwise non-host plant. Consequently, it is theoretically possible that new virus epidemics could result from heteroencapsidation (Fuchs and Gonsalves 2007). Studies in transgenic herbaceous plants showed that the expressed CP subunits are able to encapsidate the RNA genome of challenging viruses (Osburn *et al*. 1990; Holt and Beachy 1991; Candelier-Harvey and Hull 1993). Heteroencapsidation was reported for transgenic *Nicotiana benthamiana* plants expressing the CP of *Plum pox potyvirus* where a non-aphid-transmissible strain of *Zucchini yellow mosaic virus (ZYMV-NAT),* after inoculation to these plants, became aphid-transmissible (Lecoq *et al.* 1993). Studies with CCMV (Greene and Allison 1994, 1996), PPV (Varrelmann *et al.* 2000), GVA and GVB (Buzkan *et al.* 2001) and TMV (Adair and Kearney 2000) showed that heteroencapsidation between transgene sequences and the infecting virus occurs. On the other hand, it has not been found to occur in transgenic vegetable plants expressing viral CP gene constructs that were tested extensively in the field over several years at different locations. In addition, for transgenic papaya and squash, no unexpected emergence of virus species with undesired characteristics was reported even 8–10 years post-commercialization (Fuchs and Gonsalves 2007). In particular, transgenic squash and melon expressing the CP gene of an aphid-transmissible strain of CMV have been tested for their capacity to trigger the transmission of an aphid-non-transmissible strain of CMV, but no heteroencapsidation occurred (Fuchs *et al.* 1998). A low rate of transmission of a non-aphid- transmissible strain of ZYMV in transgenic squash expressing the CP gene of WMV was documented, indicating the occurrence of heteroencapsidation (Fuchs *et al.* 1999).

Altogether, changes in vector specificity and host range are a single-generation, not a permanent, event because the viral genome is not affected. As a consequence, changes will not be perpetuated in the virus progeny. Therefore, heteroencapsidation in transgenic plants expressing virus CP genes has been of limited significance and would be expected to be negligible in regard to adverse environmental effects (Fuchs and Gonsalves 2007).

The phenomenon called synergy refers to a form of complementation that occurs when plants are infected with more than one virus. The interaction of a viral protein product with another challenge virus can result in increased symptom severity and an increase in virus titer that neither virus can cause independently. In a transgenic

plant, expression of viral genes can protect against infection by a homologous virus but can also increase the susceptibility to a synergistic heterologous virus and affect the rate of disease spread. However, it does not modify existing viruses or create novel viruses with new characteristics (Fuchs and Gonsalves 2007). Plants infected with *Potato virus X* (PVX) and any other *potyviruses* show very severe symptoms and a very high PVX titre (Vance 1991). It was proposed that this phenomenon is due a mechanism close to post transcriptional gene silencing. However, the significance of synergism is limited, because it is not deemed to cause any environmental hazard.

2.4.2 ADAPTED CONSTRUCT DESIGN

It is possible that a combination of heteroencapsidation and template switching in released CP-transgenic plants could lead to the formation of “new” viruses with altered vector and host ranges and new combinations of genes (de Zoeten 1991). However, for heteroencapsidation or recombination to occur and become significant, the following sequence of different events needs to be fulfilled successfully: (1) suitable vectors need to probe or feed on susceptible transgenic host plants and transmit virus particles; (2) virions need to disassemble, the genome of challenge virus isolates needs to replicate and interact with transgene-derived products for heteroencapsidation or template switching to occur; (3) heteroencapsidated RNA molecules need to assemble and recombinant RNA molecules need to be encapsidated; (4) subsequently, heteroencapsidated and recombinant virions need to move from cell-to- cell and through the vascular system to cause systemic infection and finally (5) virions need to be acquired by vectors and transferred onto new host plants. Each step of this cascade of events requires a relatively reasonable probability of occurrence in order for a viable heteroencapsidated virus or a viable recombinant virus to develop and start an outbreak. Several constraints associated with each of these steps will reduce the success of the final outcome (Fuchs and Gonsalves 2007). The commonly used way of eliminating the potential risks associated with heteroencapsidation is the modification of the CP gene in order to prevent protein-self-assembly. So far antisense, untranslatable and truncated CP sequences have been used in experiments and different results were obtained. A reduced recovery of recombinant viruses in transgenic plants expressing a CP of *Cowpea chlorotic mottle virus* with deletions in the 3’untranslated region of transgene (Greene and Allison 1996), the suppression of particle assembly in transgenic plants expressing a mutated CP of PPV (Varrelmann and Maiss 2000), the inability to form

particles of a CP defective TMV strain (Bendahmane *et al*. 1997) and the resistance to viral infection of transgene tobacco plants transformed with a gene encoding a truncated CP of the *Tomato black ring nepovirus* (Pacot-Hiriart *et al.* 1999) have been reported. Several efforts were made to estimate resistance- and safety-aspects of transgenic grapevines using constructs containing truncated or non-translatable (Gölles *et al.* 2000, Maghuly *et al.* 2006) or antisense (Gribaudo *et al.* 2003, Gambino *et al.* 2005) sequences of the coat protein of GFLV gene. Transgenic *Nicotiana bethamiana* plants, carrying untranslatable sense-RNA or the CP sequence of GFLV in antisense orientation, showed delayed symptom development and immunity to mechanical infection with GFLV (Schlangen 2000).

Given the severe damaging impact of viruses, the strong demand for a reduction of toxic agrochemicals used for virus vector control, the pledge for a safe and sustainable agriculture and the success of biotechnologies offering alternatives to current control strategies, there is a wide range of opportunities for practical use of virus-resistant transgenic plants. In addition, the 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 (Fuchs 2003).

Risk assessment studies are needed with transgenic plants and the information on the occurrence of heteroencapsidation in transgenic herbaceous plants highlights the need for more research in this field. On the other hand, several field environmental safety assessment studies have provided strong evidence of limited environmental risks due to heteroencapsidation beyond background events (Fuchs et al 1998; 1999, 2000; Vigne et al 2004a, 2004b). There is little evidence, if any, to conclude that transgenic plants expressing viral genes alter the properties of existing virus populations or create new viruses that otherwise could not emerge in conventional plants subjected to multiple virus infection (Falk and Bruening 1994). In order to understand the significance of environmental risks, the occurrence of heteroencapsidation and recombination in the absence of transgenic plants needs to be taken into account and considered as baseline information (Fuchs and Gonsalves 2007). Adapted construct design which takes in consideration every possible risk from the very beginning on and tries to avoid it, will surely ease further development steps and help to convince also the public of the benefits of PDR, especially CPmR.

3. OBJECTIVES

The main aim of this work is to determine whether truncated forms of the GFLV CP-sequence expressed in grapevine, maintain their capacity of self-assembling, and thus represent a potential for heteroencapsidation.

Grapevine fanleaf virus (GFLV) is still one of the most destructive and wide-spread viral diseases affecting grapevine (Bovey *et al.* 1980). Since conventional control mechanisms are either expensive, laborious and dangerous for the environment or have little effect, resistance breeding seems to be a very promising strategy. The use of pathogen-derived- resistance (Lomonossoff 1995), especially coat-protein-mediated-resistance as a virus-disease-control-strategy has been reported by numerous scientists and seems to provide a very strong virus protection. The expression of the full length coat-protein sequence in a translatable orientation may however involve problems due to possible protein-protein-interactions of the transgenic coat protein. Heteroencapsidation is a phenomenon where the genome of one virus is entirely or partially encapsidated by the coat protein of another virus. The possibility of heteroencapsidation in transgenic grapevines due to interactions of a challenging virus with the transgene-encoded coat protein should be considered. It might represent a risk by leading to changes of the biological properties of the virus like vector specificity, host range and pathogenicity. To achieve acceptance for genetically modified grapevines, possible risks must be limited by the use of safe transgene-constructs which e.g. contain a mutated form of the CP gene in order to suppress particle assembly, heterologous encapsidation and complementation (Balázs and Tepfer 1997, Varrelmann and Maiss 2000).

Four different constructs, containing truncated forms of the GFLV CP-sequence, will be tested for their ability to form stable protein-protein-interactions when expressed in grapevine. The formation of stable VLPs due to the capacity of self-assembly of the expressed transgenes will be examined by the electron microscopy using ISEM technique. This electron microscopy technique involves immune-trapping of the virus on a solid support, further immune-labelling of the immobilized particles and finally observation of the results in the EM. The four translatable constructs contain one full length sequence of the coat protein, one CP-sequence that lacks 56 amino acids at

the C-terminal-end, one CP-sequence that lacks 46 amino acids at the N-terminal-end and one CP sequence with an internal deletion of 15 amino acids.

The present work will be divided into two chapters:

1. Optimization of ISEM - protocol for routine detection of VLPs in transgenic grapevines expressing CP-sequences.
2. Screening of transgenic *in vitro* and *in vivo* grapevine lines for VLPs.

In ISEM parameters vary for every virus-plant combination, thus have to be optimized previously. For the detection of GFLV an adequate protocol exists, but previous studies showed that the detection of VLPs is more complicated. In a preliminary study about the importance of truncated proteins in the process of protein folding and self-assembly, the same truncated CP constructs were transformed into *N. benthamiana*, but no VLPs were produced (Castellano and Laimer, unpublished data). It also pointed out some difficulties concerning the detection of VLPs, due to an unexpected high background. If already in herbaceous host plants the background is disturbingly high, similar, if not worse, results are expected for the woody plant. This makes clear that first of all the method has to be optimized in order to obtain interpretable results.

In the second part of this work transgenic *in vitro* and *in vivo* grapevines will be screened for the formation of VLPs with the optimized protocol. By answering the question whether the truncated coat proteins maintain the capacity of self-assembly or not i.e. if empty capsids are formed or not, the risk of heteroencapsidation in these transgenic plants will be estimated. It should be pointed out which of the analysed transgenic lines satisfies best the safety requests by not producing empty capsids, in order to propose these plant lines for further breeding studies and/ or in-field experiments.

4. MATERIALS AND METHODS

4.1 PLANT MATERIAL

In the Plant Biotechnology Laboratory (BOKU, IAM, Vienna) embryogenic cultures of *V. vinifera* (Russalka - selfpollinated) were transformed with *Agrobacterium tumefaciens* strain LBA 4404 containing various binary plasmids, pBinGUSint which carries the β-glucuronidase (GUS) marker gene, and several different constructs containing the modified coat protein genes of grapevine fanleaf virus (Gölles *et al.* 1997, 2000). The different constructs of the CP of GFLV include the full-length CP gene, truncated forms (at the 3'-, in the middle and at the 5'- end) and non-translatable forms of the CP gene either in sense or antisense orientation (Gölles 1994) and correct frame insertion of the different constructs was verified by sequencing. Previously, 127 putatively transgenic individuals of *Vitis vinifera* cv. Russalka were characterized by PCR, Southern hybridization, RT-PCR and ELISA (Maghuly *et al.* 2006). Detection of transgenic sequences by PCR was positive in all lines and Southern blot analysis revealed that the number of inserted T-DNA copies ranged from 1 to 6. Although RT-PCR analyses showed that the GFLV CP mRNA was expressed at variable levels, ELISA performed on leaf tissue did not show any accumulation of the GFLV CP in the 39 transgenic lines analyzed (Maghuly *et al.* 2006).

Ten transgenic lines expressing different translatable sequences of the GFLV coat protein were selected and used for this study of the virus-like-particle formation. Table 4 lists the studied lines and gives a short overview of their molecular characteristics. Several different plant lines containing one of the four constructs (pGA-CP+; pGA-CP; pGA-5'TR and pGA-3'TR) were screened for VLPs by the electron microscope. Figure 1 shows the different GFLV CP constructs (Gölles 1994) carried by the 10 analyzed transgenic lines. Plasmid pGA-5'TR carries a CP cDNA which is shortened by 138 bp at the 5'-end and pGA-3'TR contains a CP gene with a truncation of 168 bp at the 3'-end of the gene. Plasmid pGA-CP+ carries the full-length CP gene (1518 bp) of GFLV with an introduced start codon and pGA-CP differs from the former by a deletion of 15 bp within the CP gene corresponding to the nt 238–252 of the CP gene of GFLV strain F13 (Serghini *et al.* 1990).

The herbaceous host plants used for the preliminary study are *Ch. quinoa*, previously infected with purified GFLV by mechanical inoculation. The plants showed typical mosaic symptoms and were used at an age of about 3 weeks.

The transgenic in vitro plantlets used in this study are grown on MS0 medium in a growth chamber at 24°C and a light period of 8 hours with an intensity of 5000 lux.

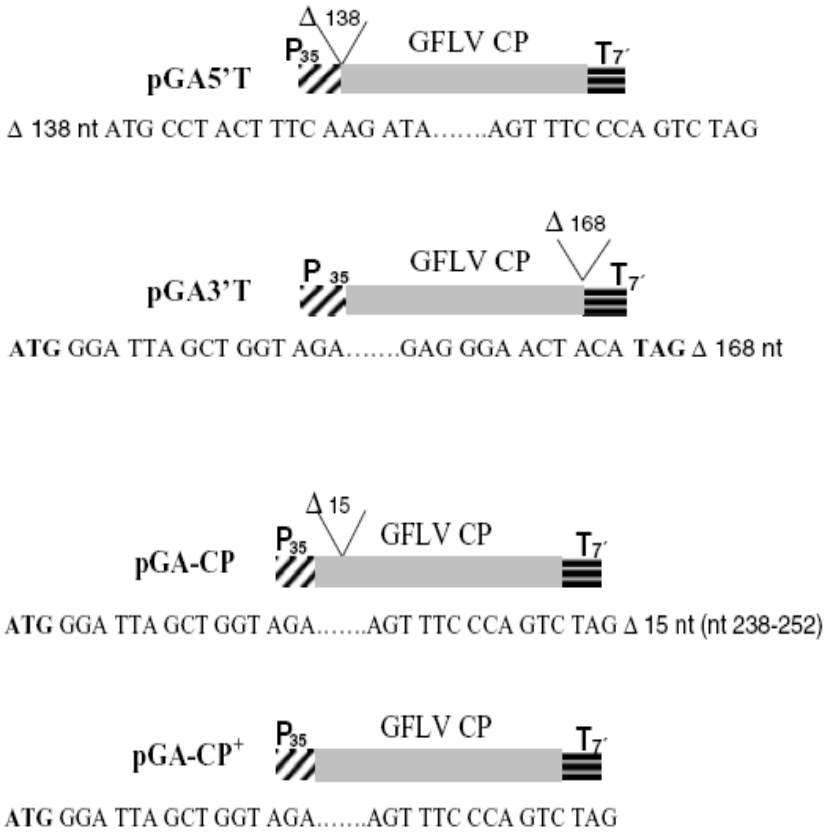

Figure 1 Expression cassettes of plant transformation vectors carrying different sequences of the GFLV-CP gene. Plasmid pGA-5'TR carries a CP cDNA which is shortened by 138 bp at the 5'-end and pGA-3'TR contains a CP gene with a truncation of 168 bp at the 3'-end of the gene. Plasmid pGA-CP+ carries the full-length CP gene (1518 bp) of GFLV with an introduced start codon and pGA-CP differs from the former by a deletion of 15 bp within the CP gene (Maghuly *et al*. 2006).

Plant group	Transformed plant lines	PCR			Copy number		ELISA
		CP	*npt*II	TET	CP	*npt*II	
1	pGA-3'TR 10.7	+	+	-	2	3	-
	pGA-3'TR 10.17	+	+	-	2	3	-
	pGA-3'TR 10.47	+	+	-	2	3	-
2	pGA-3'TR 10.39	+	+	-	1	2	-
4	pGA-3'TR 10.13	+	+	-	3	5	-
5	pGA-3'TR 10.19	+	+	-	3	4	-
	pGA-3'TR 10.41	+	+	-	3	4	-
	pGA-3'TR 10.45	+	+	-	3	4	-
6	pGA-5'TR 5.2	+	+	-	1	1	-
	pGA-5'TR 5.2	+	+	-	1	1	-
9	pGA-5'TR 5.39	+	+	-	2	1	-
11	pGA-5'TR 5.46	+	+	-	1	2	-
17	pGA-CP 4.23	+	+	-	4	2	-
22	pGA-CP+new 2.8	+	+	-	1	1	-
24	pGA-CP+new 2.6	+	+	-	6	6	-
	pGA-CP+new 2.7	+	+	-	6	6	-
	pGA-CP+new 2.54	+	+	-	6	6	-

Table 4 Characterization of the plant lines by PCR, Southern blot and ELISA using the *nptII* (kanamycin resistance gene), the TET (tetracycline resistance gene) and the CP coding region as a probe. Plants are numbered according to their origin. pGA-3'TR contains a CP gene with a truncation of 168 bp at the 3'-end of the gene. pGA-5'TR carries a CP cDNA which is shortened by 138 bp at the 5'-end. pGA-CP differs from the former by a deletion of 15 bp within the CP gene corresponding to the nt 238–252 of the CP gene of GFLV. pGA-CP+ carries the full-length CP gene (1518 bp) of GFLV (Maghuly *et al*. 2006).

ACCLIMATISATION OF *IN VITRO* PLANTLETS

Due to the high background obtained with the *in vitro* plantlets and the theory that the *in vitro* culture is responsible for this background, it was decided to try *in vivo* sample material. Some plantlets of chosen plant groups (see table 5) were acclimatized by the following procedure and afterwards used as sample material.

Plantlets were taken out of the sterile tubes, medium was carefully removed with hand-warm water and they are planted in soil. They are still protected against the environment by placing them in suitable glass containers that are closed with a plastic film. Inside these containers a micro-atmosphere is created so that there is no need of irrigation. The plants are grown at defined conditions in a growth chamber at 24°C and a light period of 8 hours with an intensity of 5000 lux. After one week the plastic film is perforated and the plantlets are carefully watered. Special caution has to be paid not to overwater the plantlets, because the newly formed roots are not capable of dealing with too much water and easily rot. If the plantlets are developing well and have no withered leaves the plastic film is removed and after another week the plantlets can be planted in small pots with normal soil. To ensure the acclimatisation went well the plantlets are kept another week in the growth chamber with the conditions described above and are then brought out into the glasshouse.

For sample material leaves, not lignified shoots and roots of the *in vivo* plantlets are used.

Plant group	Plant line	Number of plants acclimatised
1	3'TR 10.7, 10.17	3
5	3'TR 10.41, 10.45	3
11	5'TR 5.46	1
17	CP 4.23	1
22	CP+ new 2.8	1
24	CP+ new 2.7, 2.54	1

Table 5 Acclimatization of transgenic lines as characterized by Maghuly *et al.* (2006).

4.2 ISEM

GRIDS

EM grids are discs of fine mesh 3 mm in diameter, available in different materials and mesh, but best to use are 400-mesh grids (16 bars/mm) which give openings of about 40-50 µm in diameter. For short incubation periods (up to 30 minutes) and a buffer pH around 7 copper, copper/rhodium or copper/palladium grids can be used, while for incubations that last longer than half an hour more inert nickel grids are recommended (Milne 1993).

Cu/Ag grids (300-mesh, 3.5mm diameter)

Fill a wide beaker with dH_2O and gently place the plastic support with the carbon - film upwards in it so that the carbon film swims on the water surface. Take a grid with the forceps and place it under the carbon film in order to cover the grid with the film. The carbon film is placed on the Ag-side of the grid. Let them dry for several hours on filter paper.

SUPPORT FILMS

Unfortunately there is little understanding of the process of attachment, subsequent binding and orientation of antibodies, virus particles, impurities and blocking reagents to support films. A good support film should be thin enough to give a high resolution and good contrast, but robust enough to withstand a number of handling and washing steps. The two commonly used materials for support films are plastic (based on polyvinylformal or nitrocellulose) and/or carbon. Since plastic films distort under the electron beam and show poor adhesive properties for virus particles, they are normally combined with carbon which is stable under the electron beam but alone tends to brittle when grids are manipulated (Milne 1993).

However, freshly carbon coated grids were used in this experiments, because their preparation is easy and they show good adhesive properties.

BUFFERS

Several buffers can be applied successfully in ISEM, but the optimal buffer and pH range requirements can differ for every virus-host combination. The most recommended and used buffer for virus- and sera-dilution with woody species (grapevine, citrus, plum, apricot etc.) is 0.1 M phosphate buffer at a pH of 7.2 (Milne 1993).

PO_4 (Na/K) 0.1M pH 7.2

Stock solution 1: 0.1M Na_2HPO_4 (MW = 141.96 g/mol)
Stock solution 2: 0.1M KH_2PO_4 (MW = 136.09 g/mol)
Prepare 0,1M Stock solution 1 by dissolving 2.84g Na_2HPO_4 in 200ml dH_2O and 0.1M Stock solution 2 by dissolving 1.36g KH_2PO_4 in 100ml dH_2O.

Mix KH_2PO_4: Na_2HPO_4 in a ratio of 2.85 : 7.15

For 100ml PO_4-buffer 28.5ml of stock solution 2 with 71.5ml of stock solution 1are mixed.

Washing buffer: PO4 (Na/K) 0.1M pH= 7.2

Extraction Buffer: PO_4 (Na/K) 0.1M pH= 7.2 plus some drops of Nicotine (99%) as an antioxidant.

VIRUS SAMPLE PREPARATION

Normally a 100-1000 mg sample of infected tissue is homogenized with buffer. The amount of buffer added depends on the characteristics of the sample tissue. The crude extract should not be too dense in order to pipette it with a micropipette. A small amount of washed 600-mesh carborundium to facilitate the homogenisation may be added. The resulting crude extract can be used directly or as clarified supernatant after a few minutes of centrifugation at low speed.

ANTISERUM PREPARATION

The antibody preparation should be of high specificity, high avidity and high titer, but for ISEM high specificity and avidity are less imperative than for other techniques. No conjugation step that weakens the antibody avidity is required and therefore also crude antisera or purified IgG from early bleedings (two to three weeks after the first injection) can be used (Milne and Lesemann 1984).

Rabbit anti - GFLV (Aq7) serum with titer 1:512

Dilution is done with the PO_4-buffer 0.1M pH 7.2.

The antiserum was produced and kindly provided by Dr. D. Boscia at the Dipartimento di Protezione delle Piante e Microbiologia applicata in the Laboratorio di Virologia vegetale - Sezione Bari, University of Bari, Italy.

NEGATIVE STAIN

In order to visualize the decorated antibody, the preparation is rinsed with the aqueous solution of 2% Uranyl acetate. It is very important to employ an adequate washing step with double distilled water before staining, otherwise the stain will form precipitates.

OBSERVATION

The observation in the electron microscope was done with the kind assistance of Prof.ssa M. Castellano at the Dipartimento di Protezione delle Piante e Microbiologia applicata, Facoltà d'Agraria, University of Bari, Italy with a TEM Philips Morgagni. Every single grid is completely screened in order to make sure that the result is absolutely correct.

4.2.1 OPTIMIZATION OF WORKING PROTOCOL

To perform ISEM it is necessary to check the quality of the antiserum and to stabilize the parameters most suitability of the procedure. The verification of the antiserum-quality was carried out with GFLV infected *Ch. quinoa*. Afterwards the working protocol was optimized by examining of the formation of VLPs by ISEM in transgenic *in vitro* grapevines. Different antiserum dilutions for coating and trapping were investigated in order to find the optimal one and the necessity of a purification step is valuated.

PRELIMINARY TESTS WITH *CH. QUINOA*

Two or three young leaves of 3 week old plantlets of *Chenopodium quinoa*, previously infected with purified GFLV, displaying mosaic symptoms were used as sample material.

The freshly cutted leaves were homogenised in 0.1 M phosphate buffer pH 7.2 and the grids were directly incubated for 5 minutes with the resulting crude extract. After the washing with buffer for two minutes, the grids were incubated on the diluted antiserum for 15 minutes at room temperature. Four different antiserum dilutions (1:10; 1:100; 1:200 and 1:400) were used. The grids were then thoroughly washed with distilled water and stained by incubation on 2% UA for 30 seconds. The grids dried on air and were examined under the EM.

TESTS IN TRANSGENIC *IN VITRO* GRAPEVINES

Sample material: leaf tissue of transgenic *in vitro* grapevines expressing different GFLV-CP-constructs (table 6).

Procedure:

About 100 mg of the sample material is homogenized in extraction-buffer with the addition of some 600-mesh carborundium.

Grids are coated with antibody by incubating them for 1h at 37°C with the diluted antibody (1:500 or 1:300, two different dilutions are used to determine the best one).

Grids are washed twice with washing-buffer for 15 minutes and the virus is trapped by incubating the grids on the crude extract for 48h at 4°C.

Grids are washed twice with washing-buffer for 10 minutes.

The trapped virus particles are decorated with antibody by incubating the grids on the diluted antibody for 15 minutes at room temperature. In order to determine the best combination of dilutions the following combinations were used for trapping/decoration: 1:500/1:50, 1:300/1:30, 1:300/1:20.

Grids are washed thoroughly with distilled water for 10 minutes.

Grids are incubated on 2% UA for 30 seconds for staining.

Grids are let dry on air and examined under the EM.

Plant group	Plant line	Working code	Grids prepared
1	3'TR 10.7	3	5
2	3'TR 10.39	2	5
4	3'TR 10.13	1	10
5	3'TR 10.41	4	5
6	5'TR 5.2	5	5
9	5'TR 5.39	6	5
11	5'TR 5.46	7	5
17	CP 4.23	CP	5
24	CP+ new 2.6	CP+	5
3309 15 GFLV	Positive control	+K	5

Table 6 *In vitro* transgenic grapevines used as sample material. Plant groups 1, 2, 4 and 5 express a CP-sequence truncated at the 3'-end, plant groups 6, 9 and 11 express a CP-sequence truncated at the 5'-end, plant group 17 expresses a CP-sequence with an internal amino acid-deletion, while plant group 24 expresses the full length CP-sequence (Maghuly *et al.* 2006). GFLV infected *in vitro* grapevine 3309/15 GFLV is used as a positive control.

4.2.2 SCREENING OF TRANSGENIC *IN VITRO* AND *IN VIVO* GRAPEVINES FOR VLPS

Sample material: leaves or not lignified roots and shoot - tissue of transgenic *in vitro* grapevines and leaves or not lignified shoot - tissue of transgenic *in vivo* grapevines (table 8) expressing different GFLV-CP-constructs.

Procedure:

Between 100 and 1000 mg of sample material are homogenized in extraction-buffer with the addition of some 600-mesh carborundium.

The obtained crude extract is clarified by filtrating it across four layers of gauze filter tissue and subsequent centrifugation at 10000 rpm for 5 minutes.

Only the liquid and clear supernatant is used as sample – extract.

Grids are coated with antibody by incubating them for 1h at 37°C with the diluted antibody (1:300).

Grids are washed twice with washing-buffer for 15 minutes and the virus is trapped by incubating the grids on the crude extract for 24h and 48h at 4°C.

Grids are washed twice with washing-buffer for 10 minutes.

The trapped virus particles are decorated with antibody by incubating the grids on the diluted antibody (1:20) for 15 minutes at room temperature.

Grids are washed thoroughly with distilled water for 10 minutes.

Grids are incubated on 2% UA for 30 seconds for staining.

Grids are let dry on air and examined under the EM.

Plant group	Plant line	Working code	Grids prepared
In vitro			
1	3'TR 10.7, 10.17, 10.47	3a, 3b,3c	45
2	3'TR 10.39	2	18
4	3'TR 10.13	1	20
5	3'TR 10.41, 10.19, 10.45	4a, 4b, 4c	70
6	5'TR 5.1, 5.2	5a, 5b	10
9	5'TR 5.39	6	5
11	5'TR 5.46	7	5
17	CP 4.23	9	20
22	CP+ new2.8	8d	32
24	CP+ new 2.6, 2.7, 2.54	8a, 8b, 8c	25
In vivo			
1	3'TR 10.7, 10.17	3a, 3b	27
5	3'TR 10.41, 10.45	4a, 4c	15
11	5'TR 5.46	7	10
17	CP 4.23	9	5
22	CP+ new 2.8	8d	5
24	CP+ new 2.7, 2.54	8a, 8b	21
Local with GFLV infected grapevine	Positive control	MF, YM	10

Table 8 Transgenic *in vitro* and *in vivo* grapevines used as sample material for ISEM with optimized parameter. Plant groups 1, 2, 4 and 5 express a CP-sequence truncated at the 3'-end, plant groups 6, 9 and 11 express a CP-sequence truncated at the 5'-end, plant group 17 expresses a CP-sequence with an internal amino acid-deletion, while plant group 22 and 24 expresses the full length CP-sequence. Plants group 22 carry 1 copy of the transgene, while plants of group 24 carry 6 copies of the CP-sequence (Maghuly *et al.* 2006). For the positive control two local infected grapevines, one showing mosaic-symptoms (YM) and the other one showing malformation-symptoms (MF), are used as a positive control.

5. RESULTS

The polyhedral GFLV particles with a diameter of 28nm consist of 60 subunits of the coat protein. In purifications of infected plant material three types of serologically indistinguishable particles with different density properties are found. The particles were named according to their position in the sucrose density gradient: T (top) - particles, M (middle) - particles and B (bottom) - particles. The T - particles represent the empty shells consisting only of coat protein, while the M - particles contain RNA2 and the B - particles contain RNA1 and RNA2 (Quacquarelli *et al.* 1976). Since the native virus consists of three types of particles different staining results appear (figure 1). T-particles, which do not contain RNA, are penetrated by the stain and therefore visible as light round structures with dark central spot surrounded by the dark halo of the decorating antibody. The RNA contained in the M - and B - particles prevents the penetration of the stain and therefore these particles give an image that lacks the dark spot in the middle of the light round structure.

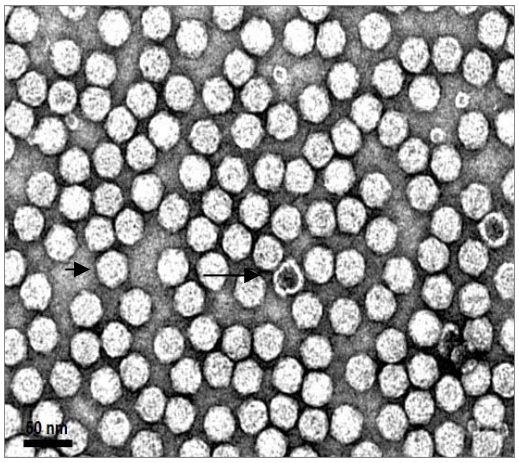

Figure 1 The negatively stained GFLV T-particles, which are penetrated by the stain, show a dark central spot (long arrows). The M - and B – particles (short arrows) appear white, because the RNA prevents the penetration of the stain into the particles (Description of plant viruses, dpv-number 385). The bar corresponds to 50 nm.

Virus-like particles (VLPs) are formed by the self-assembly of envelope and/or capsid proteins from many viruses. Such VLPs have structural characteristics and antigenicity similar to the donor virus (Grgacic and Anderson 2006), but lack viral nucleic acid, meaning that they are not infectious. Transgenic plants expressing the full length sequence of the coat protein showed the formation of VLPs (Bertioli *et al.* 1991, Spielmann et al. 2000b). Since VLPs are empty virus shells without nucleic acid, they appear with the same staining pattern as T - particles in the EM.

5.1 OPTIMIZATION OF ISEM

FIRST QUALITY CHECK: SPECIFICITY OF THE ANTISERUM

The quality of the antiserum was tested with crude extract of mechanically with GFLV infected *Chenopodium quinoa.* The grids were directly incubated with freshly prepared plant extract of the infected *Ch. quinoa,* followed by incubation in the appropriate antiserum dilution. Two grids, respectively, were prepared for the antiserum dilutions 1:10, 1:100, 1:200 and 1:400 and observed under the EM. On every grid negatively stained GFLV virus particles were detectable (figure 2).

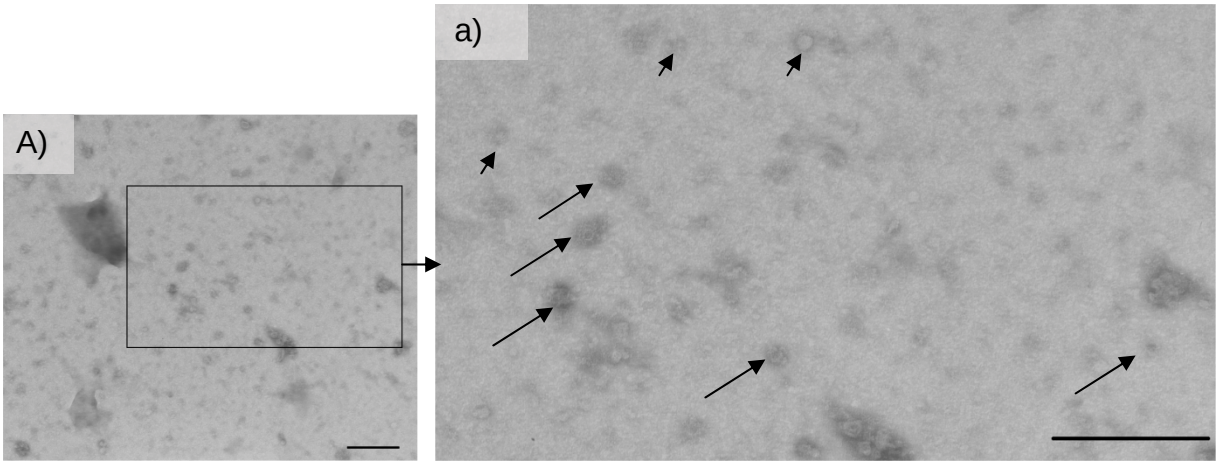

Figure 2 A) Decoration test with GFLV infected *Ch. quinoa*. a) Magnification of selected area of A). The arrows indicate negatively stained virus particles on a grid prepared with GFLV infected *Ch. quinoa* and an antiserum dilution of 1:100. The T-particles (long arrows) appear as light round structures with a dark spot in the middle and surrounded by the dark halo of antibodies. The few B-particles (short arrows), which due to the presence of RNA in the particle do not allow the stain to penetrate, appear as light, round structures surrounded by the dark halo. The bars correspond to 300nm.

Antiserum dilution	Number of analysed grids	result
1:10	2	+
1:100	2	+
1:200	2	+
1:400	2	+

Table 9 Results of the decoration test of *Ch. quinoa* infected with GFLV. Two grids were prepared for every antiserum dilution (1:10, 1:100, 1:200 and 1:400) and observed under the EM. Both grids for all antiserum dilutions resulted positive, demonstrating negatively stained GFLV particles of the T - type in an abundant manner.

These results confirm the good quality and specificity of the chosen antiserum and therefore indicate its usefulness for our application.

SECOND QUALITY CHECK: PREPARATION METHOD

Initially, all grids showed a too high background to determine any virus particles. Not even the positive control resulted clean enough to distinguish GFLV particles. Three out of ten analysed plant groups showed absolutely no result. Grids of the groups 2 (3'TR 10.39), 4 (3'TR 10.13) and 9 (5'TR 5.39) were totally covered with a dense layer of cell components and therefore not analyzable. As a demonstration figure 3 shows a dirty grid, a clean but negative grid and a positive grid. The negatively stained structures found on a clearly visible area of a grid of plant group 11 can be determined as VLPs (figure 4). Grids prepared for plant groups 1 (3'TR 10.7), 5 (3'TR 10.41), 6 (5'TR 5.2), 17 (CP 4.23) and 24 (CP+ new 2.6), showed some areas clean enough to distinguish, if present, virus particles. Four groups resulted negative: group 1 (3'TR 10.7), 5 (3'TR 10.41), 6 (5'TR 5.2) and 17 (CP 4.23). Plant line CP+ new 2.6 showed only very few, but clearly distinguishable negatively stained VLPs (figure 5). Table 10 lists the results obtained for every plant group, pointing out how many of the prepared grids were actually observable.

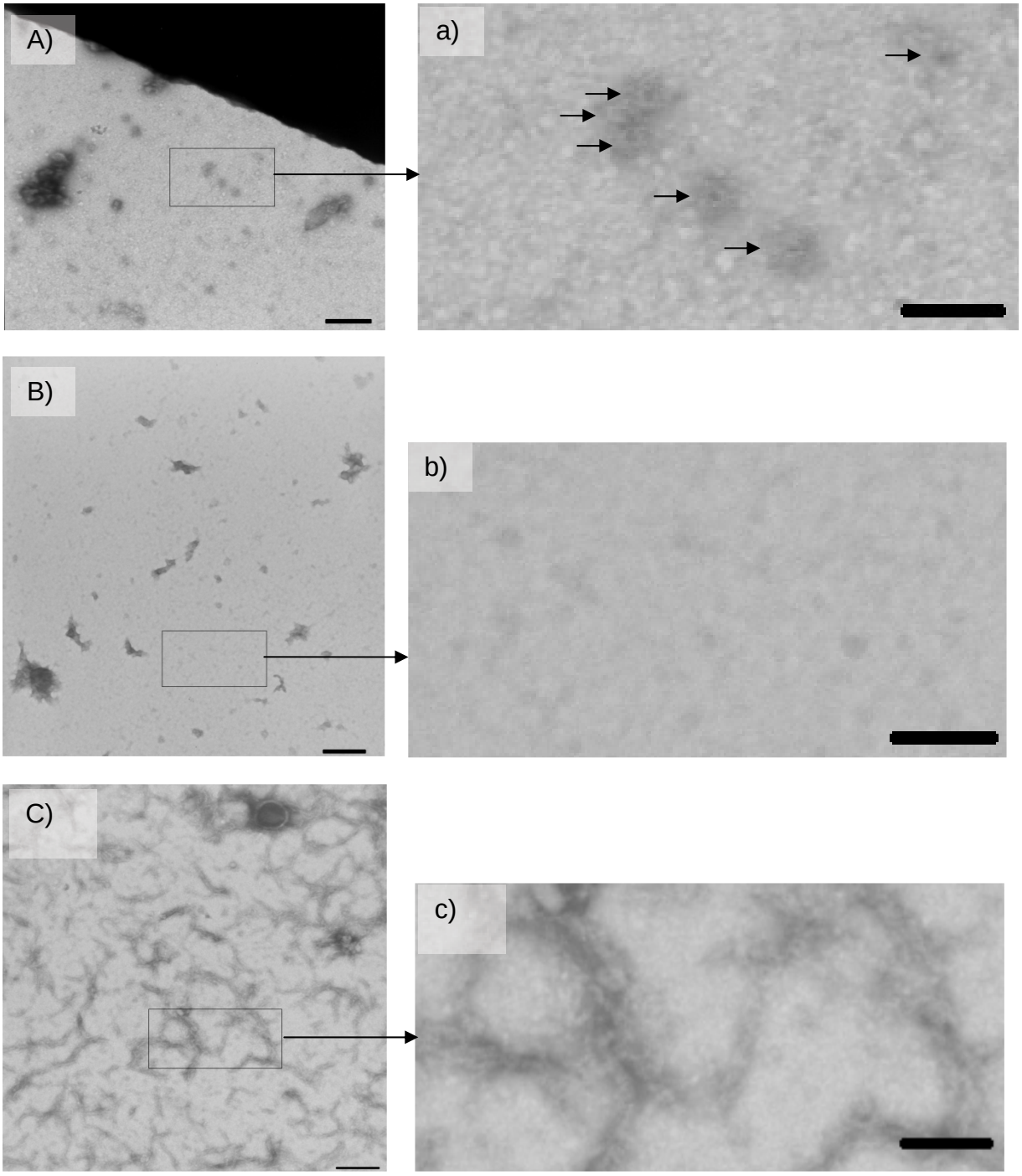

Figure 3 A) – C) Comparison of three different grids demonstrating the technical hurdles resulting from the preparation. A) positive grid showing several negatively stained GFLV - T - particles (arrows), with specific antibody halo, on a relatively clean background; B) clean and well observable grid, but without any VLPs detectable thus resulting negative; C) the whole grid is covered with fibres and proteins that make the detection of the virus particles impossible. The bars in A) – C) correspond to 300nm and in a) – c) to 150nm.

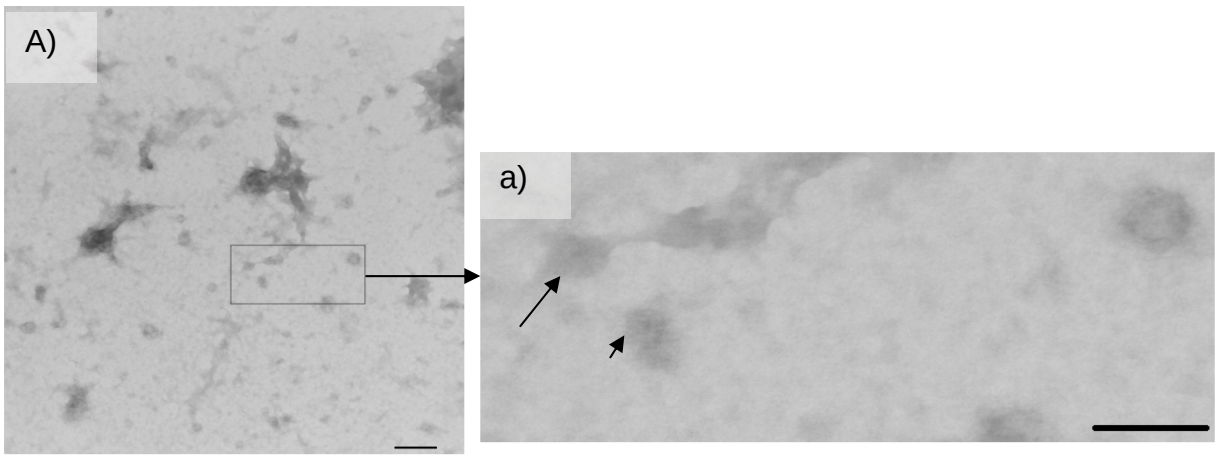

Figure 4 On a clearly observable area of a grid prepared for plant group 11 (5'TR 5.46) negatively stained, virus-like-particles of the right size (about 30 nm of diameter) and surrounded by the specific antibody halo were found. One single virus-like-structure (long arrow) and two overlapping ones (short arrow) are visible. The bar in A) corresponds to 300 nm, the bar in a) to 150 nm.

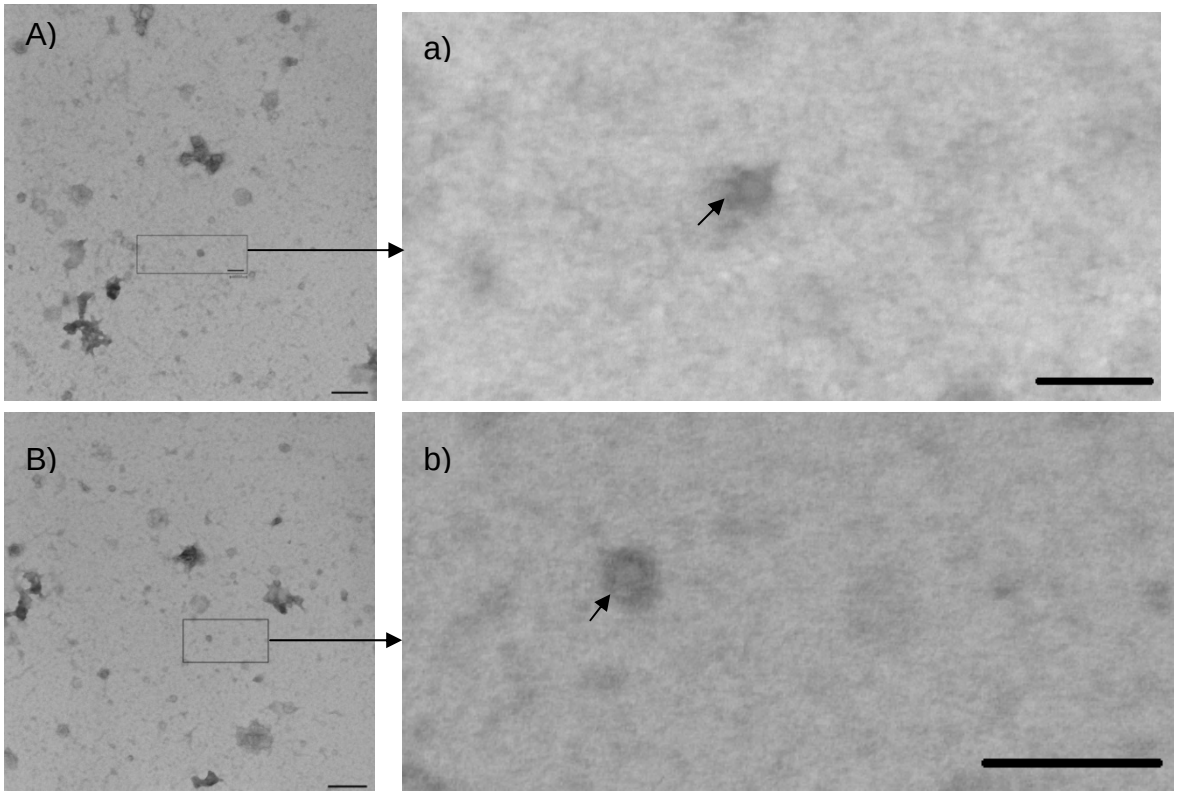

Figure 5 Plant group 24(CP+ new 2.6): on picture A) and B) a single VLP (arrow) is shown, that is stained very strongly, because many antibodies have attached to the particle. The bars in A) and B) correspond to 300 nm, the bars in a) and b) to 150 nm.

Plant group	Plant line	Results [number of grids]				
		neg.	pos.	VLP	dirty	total
1	3’TR 10.7	2	0	0	3	5
2	3'TR 10.39	0	0	0	5	5
4	3'TR 10.13	0	0	0	10	10
5	3’TR 10.41	1	0	0	4	5
6	5'TR 5.2	5	0	0	0	5
9	5'TR 5.39	0	0	0	5	5
11	5'TR 5.46	0	0	1	4	5
17	CP 4.23	2	0	0	3	5
24	CP+ new 2.6	1	0	2	2	5
3309 15 GFLV	Positive control	0	0	0	5	5

Table 10 Results of the ISEM of *in vitro* transgenic grapevines expressing different GFLV-CP-constructs. Plant groups 1, 2, 4 and 5 express a CP-sequence truncated at the 3’-end, plant groups 6, 9 and 11 express a CP-sequence truncated at the 5’-end, plant group 17 expresses a CP-sequence with an internal amino acid-deletion, while plant group 24 expresses the full length CP-sequence (Maghuly *et al.* 2006). GFLV infected *in vitro* grapevine 3309/15 GFLV is used as a positive control. The abbreviation neg. indicates grids that did not demonstrate any VLPs or virus particles; pos. indicates grids that demonstrated GFLV particles; VLP indicates grids with virus like particles and total indicates the total amount of grids prepared for that plant group.

In order to obtain better results, i.e. clearer grids with less background and more analyzable areas, the protocol was improved. The chosen parameters for the ISEM are summarized in table 11 and were subsequently used for the analyses of the transgenic grapevines. A filtration- and a centrifugation- step were added to the sample preparation procedure in order to remove as much as possible of the disturbing cell components. An additional incubation period of 24 hours was added, in order to trap enough virus particles, but to limit the attachment of disturbing cell components.

Working step	Dilution	Incubation method
Clarification of crude extract	-	Filtration with gauze; centrifugation at 10000 rpm for 5 minutes
Coating	1:300	1 hour at 37°C
Trapping	-	24 and 48 hours at 4°C
Decoration	1:20	15 minutes at RT
Negative staining	-	30 seconds at RT

Table 11 Summary of the optimized and definitive parameters for ISEM.

5.2 SCREENING OF TRANSGENIC *IN VITRO* AND *IN VIVO* GRAPEVINES

Some of the plant groups characterized by Maghuly *et al.* (2006) contain more than one plant line. The plant lines are grouped into a plant group according to their origin from the same transformation event, since they show the same pattern in Southern blot analyses (Maghuly *et al*. 2006). The adapted protocol for ISEM was used to confirm the results obtained in the first row of experiments and to analyze different plant lines belonging to the same plant group. Most prepared grids showed better results concerning the background and had several clearly observable areas. Thus VLPs should be detectable, if present. Table 12 summarizes all results obtained for the ten *in vitro* and the six *in vivo* transgenic plant lines analysed and the two GFLV infected local grapevines used as positive control.

The two infected local grapevines used as positive controls showed several negatively stained GFLV particles on all grids (figure 6). The GFLV particles are visible as light icosahedral structures of the correct size (28nm), surrounded by the typical dark halo of the stained antibodies.

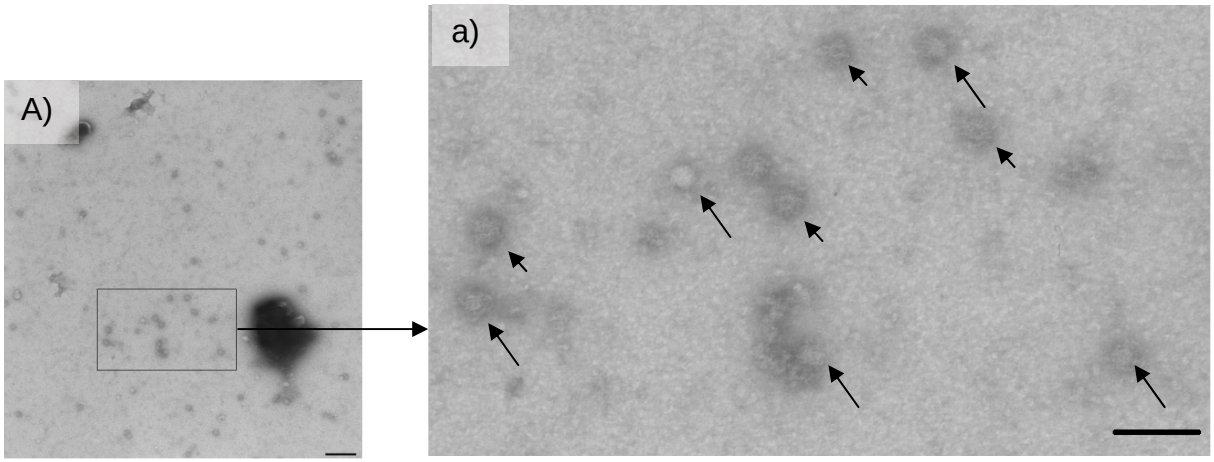

Figure 6 As a positive control for the ISEM a GFLV infected local grapevine was used. The detected virus particles are mainly of the T-type (short arrows), resulting in light particles with a dark spot in the middle and surrounded by the dark halo of specifically attached antibodies. A few B-particles (long arrows), characterized by the absence of the dark central spot, are also visible. The bar in A) corresponds to 300 nm, the bar in a) to 150 nm.

IN VITRO:

More than 50% of the grids for plant groups 1 (3'TR 10.7, 10.17, 10.47), 2 (3'TR 10.39) and 17 (CP 4.23), respectively, could be evaluated and none of them showed negatively stained structures comparable to VLPs. Therefore these groups were considered to be negative. Very clear results were obtained for all three lines of group 1 (3'TR 10.7, 10.17 and 10.47). Since 32 out of 45 prepared grids were clean and well observable and none of them carried any VLP, the negative results of the previous experiment (see section 5.1) were confirmed and supported. Plant groups 6 (5'TR 5.1, 5.2), 9 (5'TR 5.39) and 11 (5'TR 5.46) also yielded negative results, although only fewer grids were observable, compared to all plant groups. Both lines of group 6 (5'TR 5.1 and 5.2) resulted negative, although due to the few clean grids further analyses are required to obtain a final confirmation for this plant group. Since only one out of five prepared grids was observable, the negative result for plant group 9 cannot be considered as definitive. Only four out of 20 prepared grids were observable, but no VLPs were detectable for plant group 4 (3'TR 10.13).

More than half of the grids of plant group 5 (3'TR 10.19, 10.41, 10.45), 22 (CP+ new 2.8) and 24 (CP+ new 2.6, 2.7, 2.54) were clearly observable and isolated VLPs were found (figure 7). The three analysed plant lines of plant group 5 (3'TR 10.19, 10.41, 10.45) did not show consistent results. Plant line 3'TR 10.41 had a too high background to distinguish VLPs, while in plant line 3'TR 10.45 a few negatively stained VLPs (figure 7a) were detectable and plant line 3'TR 10.19 resulted negative. Three plant lines of the plant group 24 were analysed and in two of them (CP+ new 2.6 and 2.7) negatively stained VLPs were detectable. Plant line CP+ new 2.54 resulted negative, because no VLPs were detectable. An isolated, but characteristically stained, VLP is visible on a grid of plant line CP+ new 2.6 (figure 7c). Plants of group 22 carry 1 copy of the transgene, while plants of group 24 carry 6 copies of the CP-sequence (Maghuly *et al.* 2006).

Plant group 22 (CP+ new 2.8) was added to the analyses in order to answer the question whether VLP formation is linked to the number of introduced transgenes or not. The results obtained indicate that there is no correlation between number of introduced transgene and VLP formation, because the negatively stained VLPs were detectable on both the grids of plant group 22 and 24 (figure 7b and 7c).

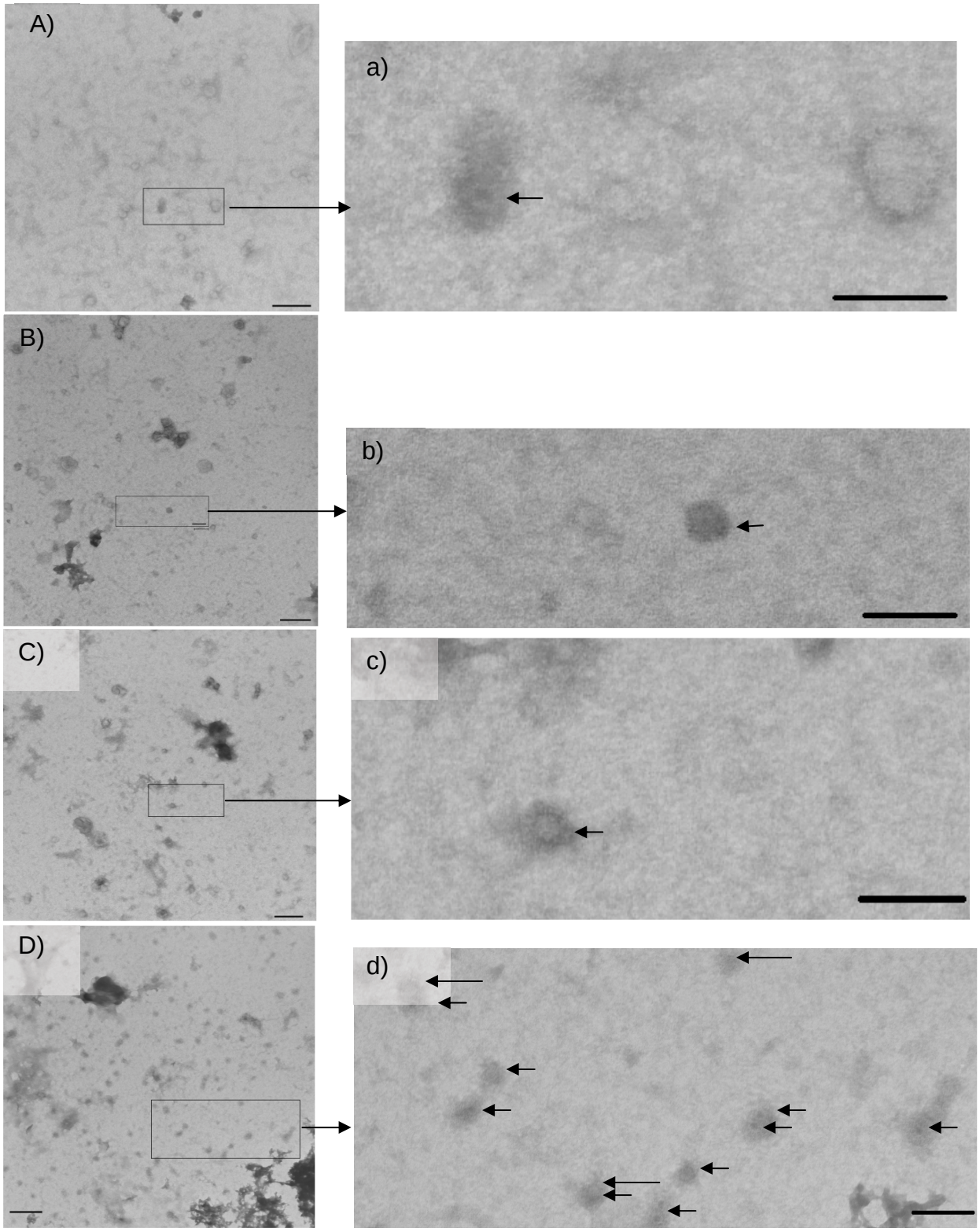

Figure 7 Isolated VLPs found in the transgenic in vitro grapevines: A) plant group 5 (3'TR 10.45); B) plant group 22 (CP+ new 2.8); C) plant group 24 (CP+ new 2.6) and D) *in vivo* grapevine infected with GFLV (positive control) showing T-particles (short arrows) and B-particles (long arrows). The bars in A) - D) correspond to 300 nm and in a) – d) to 150 nm.

IN VIVO:

Since the quality of the prepared grids was not optimal and disturbing background was thought to be a characteristic of the stressed *in vitro* plants, the decision was made to try the ISEM with transgenic *in vivo* grapevines. It was hoped that the background can be further decreased in order to obtain sample batches, where all grids were clear and observable.

The acclimatization worked well and all plants were successfully transferred from *in vitro* culture to the glasshouse conditions. The grids prepared with sample material extracted from *in vivo* plants showed nearly no background, thus confirming the assumption that the high background resulted from the *in vitro* culture.

Plant groups 1 (3'TR 10.7, 10.17), 11 (5'TR 5.46), 17 (CP 4.23) 22 (CP+ new 2.8) and also 24 (CP+ new 2.54, 2.7) demonstrated a very clear negative result. Although nearly all prepared grids for plant group 24 (CP+ new 2.54, 2.7) were clean and observable, both of the lines resulted negative, because not even a single VLP was detectable. Plant group 5 (3'TR 10.41, 10.45) showed some negatively stained VLPs. A clearly distinguishable VLP, surrounded by the characteristic halo of stained antibodies, was found in plant line 3'TR 10.45 (figure 8).

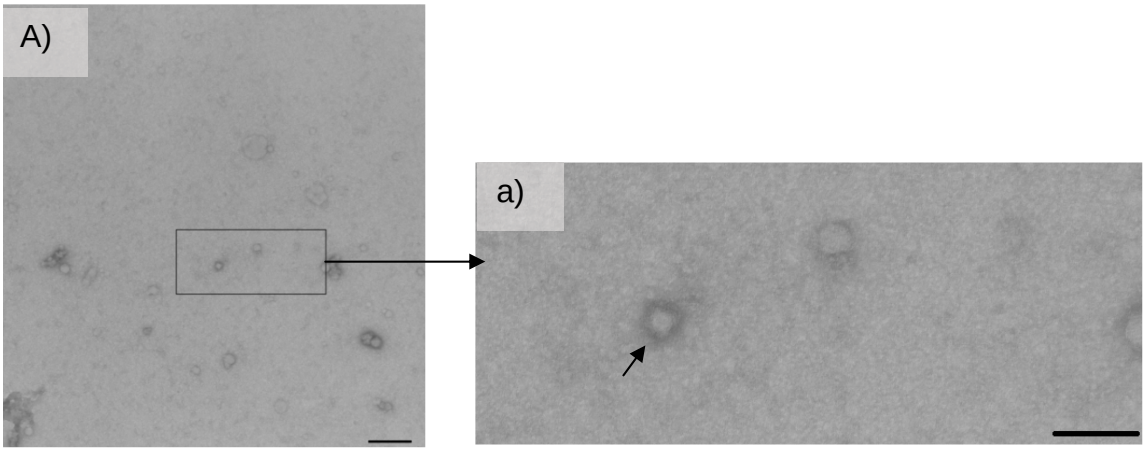

Figure 8 A clearly distinguishable VLP (arrow) on a grid prepared of *in vivo* material of plant group 5 (3'TR 10.45) surrounded by a big dark halo of antibodies. The bar in A) corresponds to 300 nm and in a) to 150 nm.

Plant group	Plant line	Results [number of grids]				
		neg.	pos.	VLP	dirty	total
In vitro						
1	3'TR 10.7	16	0	0	9	25
	3'TR 10.17	10	0	0	5	15
	3'TR 10.47	15	0	0	0	15
2	3'TR 10.39	9	0	0	9	18
4	3'TR 10.13	4	0	0	16	20
5	3'TR 10.19	5	0	0	10	15
	3'TR 10.41	13	0	0	7	20
	3'TR 10.45	22	0	3	10	35
6	5'TR 5.1	1	0	0	4	5
	5'TR 5.2	1	0	0	4	5
9	5'TR 5.39	1	0	0	4	5
11	5'TR 5.46	2	0	0	3	5
17	CP 4.23	14	0	0	6	20
22	CP+ new 2.8	25	0	1	6	32
24	CP+ new 2.6	7	0	1	7	15
	CP+ new 2.7	1	0	0	4	5
	CP+ new 2.54	3	0	0	2	5
In vivo						
1	3'TR 10.7	17	0	0	0	17
	3'TR 10.17	6	0	0	4	10
5	3'TR 10.41	5	0	0	0	5
	3'TR 10.45	9	0	0	1	10
11	5'TR 5.46	6	0	0	4	10
17	CP 4.23	4	0	0	1	5
22	CP+ new 2.8	5	0	0	0	5
24	CP+ new 2.7	8	0	0	2	10
	CP+ new 2.54	8	0	1	1	10
Positive control	YM, MF	0	10	10	0	10

Table 12 Transgenic *in vitro* and *in vivo* grapevines used as sample material for the ISEM with optimized parameters. Plant groups 1, 2, 4 and 5 express a CP-sequence truncated at the 3'-end, plant groups 6, 9 and 11 express a CP-sequence truncated at the 5'-end, plant group 17 expresses a CP-sequence with an internal amino acid-deletion, while plant group 22 and 24 expresses the full length CP-sequence. Plants group 22 carry 1 copy of the transgene, while plants of group 24 carry 6 copies of the CP-sequence (Maghuly *et al.* 2006). For the positive control two local infected grapevines, one showing mosaic-symptoms (YM) and the other one showing malformation-symptoms (MF), were used. All grids of the positive control showed both types of GFLV particles (B-particles and VLPs). The abbreviation neg. indicates grids that did not demonstrate any VLPs or virus particles; pos. indicates grids that demonstrated GFLV particles; VLP indicates grids with virus like particles and total indicates the total amount of grids prepared for that plant group.

It can be finally summarized that plant groups 1 (3'TR 10.7, 3'TR 10.47 and 3'TR 10.17), 6 (5’TR 5.1 and 5’TR 5.2) and 17 (CP 4.23) resulted clearly negative concerning the formation of VLPS, while for plant groups 5 (3'TR 10.41, 3'TR 10.19 and 3'TR 10.45.1), 11 (5’TR 5.46), 22 (CP+ new 2.8) and 24 (CP+ new 2.6, CP+ new 2.54, CP+ new 2.7) VLPs were detected.

6. DISCUSSION AND CONCLUSION

Due to the deletion of amino acids in the transgene sequence possibly interfering with protein-protein-interactions of the final translation product and the low expression rate of the transgene, the VLPs, if formed, are expected to be very unstable. Unfortunately many of the initially produced grids showed a disturbing background consisting of a more or less dense layer of plant-cell-proteins. The GFLV- particles have a diameter of 28 nm and are not distinguishable on such a background, simply due to the fact that the background particles are much larger than the virus itself. However, only because high background prevents the detection of VLPs, this does not exclude their presence. They could be hidden under or between the attached and much larger background-forming-proteins. In order to obtain more reliable results, the method was optimized and adapted to this special situation. A filtration and centrifugation step was introduced and appears recommendable, because with this small change improved results were obtained. In fact, the background on the grids decreased, so that for most plant lines more than 50 % of the prepared grids showed wide clean and observable areas. In most cases the grids incubated with the plant extract for only 24h showed more clean and observable areas, due to the fact that the remaining disturbing background proteins had less time to attach to the grid. However, for GFLV detection extremely long incubation times are needed. Best results were reported after 8 days of incubation at 4°C, when 270 times more virus was trapped than after 15 minutes (Bovey *et al.* 1980). However, in this study the incubation time of 48h was maintained to ensure that every present particle attached to the grid. The improvement and the adaptation of the protocol to the special situation were successful and led to a method that is able to yield reliable results.

The quantity and quality of the prepared samples allow the clear conclusion that this method (for the standardized ISEM procedure see appendix 8.1) is suitable for the screening for VLPs in transgenic grapevines expressing the full length or truncated coat protein of GFLV. The obstacles, due to a too high background, faced in the beginning were overcome by filtration and centrifugation of the crude plant extract prior to incubation of the grids. Incubating the samples parallel for 24 and 48 hours is

highly recommended. It means only little more work, but leads to more reliable results. The best results were still obtained with *in vivo* sample material, although also for the *in vitro* material clear results were obtained after the modification of the protocol.

The results of the experiments with the optimized protocol clearly demonstrate that the changes in the protocol are an improvement. Plant lines 2, 4 and 9, e.g., which in the first row of experiments (see section 5.2) were not analyzable due to a very high background, could be analyzed with the improved sample preparation method. However the results of plant lines 2, 4 and 9 are not final, because less than 50% of the prepared grids were observable and these plant lines are therefore not taken into considerations for the final evaluation. Further tests need to be carried out for a final judgement.

Some of the analysed transgenic grapevines resulted clearly negative concerning the formation of VLPs. Plants of line 1 (carrying a CP-sequence truncated at the 3'-terminal), plant line 6 (carrying a 5'- truncated CP-sequence) and plant line 17 (carrying a CP-sequence with an internal deletion) showed not a single VLP. One explanation could be that the transgenic grapevines failed to synthesize the CP mRNA, but this can be ruled out, since Northern blots revealed, that all lines express the transgene (Maghuly *et al.* 2006). The second explanation, that the antiserum prepared against the wild-type CP could fail to recognize the transgene CP due to the mutation, is, at least in this case, disproved by the fact that in some plant lines VLPs were detectable. The third explanation, that the protein synthesized could be destabilized by its truncations and thus could fail to self-assemble to stable VLPs (Pacot-Hiriart *et al.* 1999), yields the most convincing interpretation.

Unlike Pacot-Hiriart *et al*. (1999), who did not detect any pseudo-particles of *Tomato black ring nepovirus* by ISEM in transgenic tobacco plants expressing a truncated form of the coat protein, some VLPs in preparations of plant lines 5 (carrying the 3'-terminal truncated CP-sequence) and 11 (carrying a 5'-terminal deletion) have been detected. The VLPs, if detected, always occurred at very low frequency, i.e. one or two VLPs were detectable on 1 grid out of 5. This may be a result of the low expression rate of the transgene, but also of the decreased capacity for protein-protein-interactions, resulting in unstable particles.

As already reported for ArMV in CP-transgenic tobacco plants (Bertioli *et al.* 1991), the transgenic grapevines expressing the full length GFLV-CP-sequence (plant groups 22 and 24) produced some VLPs. The VLPs were clearly distinguishable, but very few in number.

It has to be further mentioned that there was no correlation detectable between the introduced copy number of the transgene and the formation of VLPs, because both plant group 22 (CPnew+ 2.8) and plant group 24 (CPnew+ 2.6, 2.7, 2.54) demonstrated VLPs. Although plant group 22 carries only one transgene, while plant group 24 carries six copies of the full length CP-sequence, no difference in VLP formation was detected.

Finally it has to be mentioned that it was not possible to establish a general statement, whether the formation of VLPs was more negatively influenced by the 3'-end - or the 5'- end - truncation. Plant group 1 (3'TR 10.7 10.17, 10.47), transformed with the GFLV CP gene with the 3'-end truncation, resulted negative, but plant group 5 (3'TR 10.45), carrying the same CP construct only with a different transgene copy number, showed VLPs. Comparable results were obtained for plant groups expressing the CP gene truncated at the 5'-end, where plant group 6 (5'TR 5.1, 5.2) resulted negative and plant line 5'TR 5.46 showed VLPs.

ISEM results as a very simple, rapid and secure method either for virus detection (Milne 2006) or detection of VLPs in CP-transgenic plants (Bertioli *et al.* 1991; Spielmann *et al.* 2000b). The result is obtainable within a time range of two hours to two days and false positives are really rare, because either there are negatively stained VLPs visible or not. The only disadvantage that has to be noted is that small, isometric particles, like GFLV, are commonly difficult to determine if the background is not really clean.

Transgenic plants with viral CP provide specific resistance against the viruses with identical or similar CPs (Powell *et al.* 1986; Beachy *et al.* 1990; Pacot-Hiriart *et al.* 1999; Spielmann *et al.* 2000a; Vigne *et al.* 2004b), but due to several characteristics of viruses and their replication already very early assessments of the possible dangers of the release of virus-gene-transgenic plants in the environment arose (de Zoeten 1991). Potential safety considerations relate directly to the fact that resistance to viruses in plants is achieved through constitutively expressing viral sequences,

which do not occur in healthy plants (Fuchs and Gonsalves 2007). Heteroencapsidation has been already reported for transgenic tobacco plants expressing the CP of PPV (Lecoq *et al.* 1993; Varrelmann *et al.* 2000), CCMV (Greene and Allison 1994, 1996), GVA and GVB (Buzkan *et al.* 2001) and TMV (Adair and Kearney 2000). On the other hand, for transgenic papaya and squash, no unexpected emergence of virus species with undesired characteristics was reported 8–10 years post-commercialization (Fuchs and Gonsalves 2007).

Knowledge on the real effects of virus resistant transgenic plants has expanded and two decades after their introduction, no scientific study has documented any detriment to the environment attributable to virus-resistant transgenic plants. Also, there is a documented history of safe commercial use of virus-resistant transgenic squash and papaya in the United States. Lessons from field experiments and commercial releases have provided overwhelming evidence that benefits largely outweigh risks and that virus-resistant-transgenic plants are safe for the environment and consumers (Fuchs and Gonsalves 2007).

As a final conclusion can be drawn that ISEM is suitable for the detection of VLPs in GFLV-CP-transgenic grapevines and that it is highly recommendable as a standard monitoring method for in-field experiments. The plant lines 1 (carrying the 3'- terminal truncated CP-sequence), 6 (carrying a 5'-terminal deletion) and 17 (carrying the CP-construct with the internal deletion) resulted absolutely negative, thus recommending them as perfect candidates for field experiments in order to determine the resistance against GFLV infection. As the truncated versions of the CP are not able of protein-protein-interaction, these plants result absolutely safe concerning risks such as heteroencapsidation or transencapsidation. However, due to the fact that also in plant groups that did not result clearly negative, only very few and mostly instable VLPs were found, the risk of heteroencapsidation can be considered nearly not existing also for these plant groups. Heteroencapsidation is not considered dangerous anymore, because if occurring alone it is a single-generation-event (Fuchs and Gonsalves 2007) and could be simply eliminated by stopping the cultivation of the plants (Prins *et al.* 2007). However, in order to satisfy all safety demands, the use of truncated CP-versions unable to self-assemble and the continuous monitoring for VLPs with the ISEM during the development of transgene plants, as demonstrated in this work, appears a promising approach.

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8. Appendix

8.1 PROTOCOL FOR THE ISEM

8.1.1 Material

- **Preparation of the Grids**

Material: Cu/Ag grids (300-mesh, 3.5 mm diameter)

Fill a wide beaker with dH_2O and gently place the plastic support with the carbon - film upwards in it so that the carbon film swims on the water surface. Take a grid with the foreceps and place it under the carbon film in order to cover the grid with the film. The carbon film is placed on the Ag-side of the grid. Let them dry for several hours on filter paper.

- **Buffer**

PO_4 (Na/K) 0.1M pH 7.2

Stock solution 1: 0.1M Na_2HPO_4 (Mr = 141.96 g/mol)
Stock solution 2: 0.1M KH_2PO_4 (Mr = 136.09 g/mol)

Prepare 0,1M Stock solution 1 by dissolving 2.84g Na_2HPO_4 in 200ml dH_2O and 0.1M Stock solution 2 by dissolving 1.36g KH_2PO_4 in 100ml dH_2O.

Mix KH_2PO_4: Na_2HPO_4 in a ratio of 2.85 : 7.15

Washing buffer: PO4 (Na/K) 0.1M pH= 7.2
Extraction Buffer: PO_4 (Na/K) 0.1M pH= 7.2 plus some drops of Nicotine (99%) as an antioxidant.

- **Antiserum**

Rabbit anti - GFLV (Aq7) serum

Dilution for capture: 1:300 Dilution for decoration: 1:20
Dilution is done with the PO_4-buffer 0.1M pH 7.2

8.1.2 Procedure

- **Sample material:** leaf tissues, young and not lignified shoots and roots, phloematic tissue

- **Method of incubation**

  All incubation steps are made by floating the grids face down on a drop of the respective solution placed on a hydrophobic surface like parafilm or hydrophobic filter paper placed in a humid chamber.

- **Extraction:**

  1. The weighted plant tissue is homogenized in 0.1M Extraction buffer in a mortar.
  2. Clarify the obtained homogenizate by filtrating it across four layers of gauze filter tissue.
  3. Centrifuge the clarified sap at 10000 rpm for 5 minutes.
  4. Use only the liquid and clear supernatant as sample - extract and do not touch the pellet.

- **Antibody capture:**

  5. Create a humid chamber by putting a wet Whatman filter paper in a petri dish and cover it with a hydrophobic surface (e.g. Whatman filterpaper silliconated).
  6. Place 15 µl drops of the rabbit anti - GFLV (Aq7) serum using a 1:300 dilution on the hydrophobic surface.
  7. Place the carbon-coated grids with the carbonated side down on the Antiserum and incubate them in the closed humid chamber for one hour at 37°C. Make sure that the carbon – coated side of the grid is in contact with the liquid.

- **Trapping the virus:**

8. Wash the grids twice in the PO_4-washing buffer for 15 minutes, respectively. (0.1M phosphate buffer pH 7.2).
9. Create another humid chamber and place drops of the sample extract from step 4 on the hydrophobic surface.
10. Transfer the grids on the drops of the sample - extract and incubate them at 4°C for 24 or 48 hours respectively.

- **Antibody Decoration:**

11. Wash the grids twice in PO_4-washing buffer for 10 minutes respectively.
12. Incubate the grids with rabbit anti - GFLV (Aq7) serum using a 1:20 dilution for 15 minutes at room temperature by placing them on 15 µl drops of the antiserum on the hydrophobic surface of the humid chamber.
13. Wash the grids for two minutes with dH_2O.
14. Proceed the negative staining by incubating the grids for 30 seconds on 15 µl drops of 2% uranyl acetate.
15. Gently dry the grids by placing them on clean filterpaper and observe them under the electron microscope.

8.2 Table of Results

Plant line	Working code	Plant group	Results in vitro		Results in vivo	Number of grids				
			Results 1	Results 2		positive	negative	dirty	VLP	total
3'TR 10.13.1	1.1	4	dirty	negative	-	0	4	21	0	25
3'TR 10.13.2	1.2		dirty	-	-	0	-	5	-	5
3'TR 10.39	2	2	dirty	negative	-	0	9	14	0	23
3'TR 10.7	3; 3a	1	negative	negative	negative	0	32	15	0	47
3'TR 10.47	3c		-	negative	-	0	15	0	0	15
3'TR 10.17	3b; 1b		-	negative	negative	0	10	5	0	15
3'TR 10.41	4; 4a	5	negative	dirty	negative	0	19	11	0	30
3'TR 10.19	4b		-	negative	-	0	5	10	0	15
3'TR 10.45.1	4c		-	VLP	VLP	0	31	11	3	45
5'TR 5.1	5b	6	-	negative	-	0	1	4	0	5
5'TR 5.2	5; 5a		negative	negative	-	0	7	3	0	10
5'TR 5.39	6	9	dirty	negative	-	0	1	9	0	10
5'TR 5.46	7	11	VLP	negative	negative	0	9	10	1	20
CP+ new 2.6	CP+; 8c	24	VLP	VLP	-	0	8	9	3	20
CP+new 2.54	8a		-	negative	negative	0	12	4	0	16
CP+new 2.7	8b		-	negative	negative	0	9	6	0	15
CP+new 2.8	8d	22	-	VLP	negative	0	30	6	1	37
CP 4.23	CP; 9	17	negative	negative	negative	0	20	10	0	30
3309 15 GFLV	+K	-	dirty	-	-	0	0	5	0	5
local grapevine of Puglia infected with GFLV			-	-	-	-	-	-	-	-
yellow mosaic symptoms			-	-	positive	5	-	-	-	5
malformation symptoms			-	-	positive	5	-	-	-	5

Table 13 A summary of the obtained results. The plant lines and the plant groups are characterized by Maghuly *et al.* (2006). The working code was used to facilitate the handling and quick destinction of the plants during the experiments. Results 1 of *in vitro* results are the one obtained while the optimization of the protocol, while Results 2 and Results *in vivo* are obtained using the optimized protocol. Positive are those grids with GFLV particles on them, while the one with virus like particls are termed "VLP". The total number of grids is showed to point out whether the final result for the plant line is consolidated or not.

8.3 Acronyms

Ag	Silver (Argentum)
AM	Ammonium molybtate
Aq7	Aquila 7
ArMV	*Arabic mosaic virus*
B - particles	Bottom - particles
BYMV	*Bean yellow mosaic potyvirus*
°C	Degrees celsius
CCMV	*Cowpea chlorotic mottle virus*
CMV	*Cucumber mosaic virus*
CP	Coat protein
CPmR	Coat protein mediared Resistance
Cu	copper
dH_2O	destilled water
DNA	Desoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron Microscope
et al.	et alii
FAO	Food and Agriculture Organization
g	gram
GFLV	*Grapevine fanleaf virus*
GUS	B - glucuronidase
GVA	*Grapevine virus A*
GVB	*Grapevine virus B*
h	hour
IEM	Immune Electron Microscopy
IgG	Immunoglobulin G
IR	Inverted repeat
ISEM	Immuno sorbent electron Microscopy
kb	Kilo bases
KOH	Potassiumhydroxide
M	Molar (mol/liter)

M - particles	Middle - particles
MF	Malformation symptoms
µm	micrometer
mg	milligram
ml	milliliter
mm	millimeter
Mr	Molecular weight in Dalton
mRNA	messenger RNA
MS	Murashige and Skoog - Medium
NaOH	Sodiumhydroxide
nm	nanometer
nptII	Kanamycin resistance gene
nt	nucleotide
PCR	Polymerase chain reaction
PDR	Pathogen - derived - resistance
PO_4	Phosphate
PPV	*Plum pox virus*
PTA	Phosphotungstic acid
PTGS	Posttranscriptional gene silencing
PVX	*Potato virus X*
Rep-mR	Replicase - mediated resistance
RNA	Ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT - PCR	Reverse transcription polymerase chain reaction
siRNA	small interfering RNA
T - DNA	transferred DNA
T - particles	Top - particles
TEM	Transmission Electron Microscope
TET	Tetracycline resistance gene
TMV	*Tobacco mosaic virus*
tRNA	transcription RNA
UA	Uranyl acetate
VLP	Virus like particle

YM	Yellow mosaic symptoms
ZYMV	*Zucchini yellow mosaic virus*
ZYMV-NAT	non-aphid-transmissible strain of *Zucchini yellow mosaic virus*